United States Department of Agriculture

Animal and Plant Health Inspection Service

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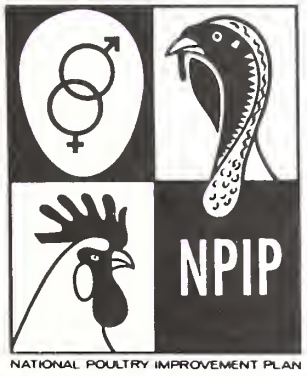
National Poultry Improvement Plan and Auxiliary Provisions



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Part 145—National Poultry Improvement Plan

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Authority: 7 U.S.C. 429; 7 CFR 2.17, 2.51, and 371.2(d).

Subpart A—General Provisions

§145.1 Definitions

Words used in this part in the singular form shall be deemed to import the plural, and vice versa, as the case may demand. Except where the context otherwise requires, for the purposes of this part the following terms shall be construed, respectively, to mean:

Administrator	The Administrator, Animal and Plant Health Inspection Service, or any person authorized to act for the Administrator.
Affiliated flockowner	A flockowner who is participating in the Plan through an agreement with a participating hatchery.
Animal and Plant Health Inspection Service	The Animal and Plant Health Inspection Service of the U.S. Department of Agriculture.
Authorized Agent	Any person designated under § 145.11(a) to perform functions under this part.
Authorized laboratory	A laboratory designated by an Official State Agency, subject to review by the Service, to perform the blood testing and bacteriological examinations provided for in this part.
Baby poultry	Newly hatched poultry (chicks, poults, ducklings, goslings, keets, etc.) that have not been fed or watered.
Colon bacilli	For the purpose of this chapter, those organisms which are gram negative, non spore-forming bacilli, which ferment lactose with gas formation, and serve as an index of fecal contamination.
Dealer	An individual or business that deals in commerce in hatching eggs, newly-hatched poultry, and started poultry obtained from breeding flocks and hatcheries. This does not include an individual or business that deals in commerce in buying and selling poultry for slaughter only.
Department	The U.S. Department of Agriculture.
Domesticated	Propagated and maintained under the control of a person.
Equivalent or equivalent requirements	Requirements which are equal to the program, conditions, criteria, or classifications with which compared, as determined by the Official State Agency and with the concurrence of the Service.
Exposed (Exposure)	Contact with birds, equipment, personnel, supplies, or any article infected with, or contaminated by, communicable poultry disease organisms.

Flock

(1) As applied to breeding. All poultry of one kind of mating (breed and variety or combination of stocks) and of one classification on one farm;
(2) As applied to disease control. All of the poultry on one farm except that, at the discretion of the Official State Agency, any group of poultry which is segregated from another group and has been so segregated for a period of at least 21 days may be considered as a separate flock.

Fluff sample

Feathers, shell membrane, and other debris resulting from the hatching of poultry.

Fowl typhoid or typhoid

A disease of poultry caused by *Salmonella gallinarum*.

Franchise breeder

A breeder who normally sells products under a specific strain or trade name and who authorizes other hatcheries to produce and sell products under this same strain or trade name.

Franchise hatchery

A hatchery which has been authorized by a franchise breeder to produce and sell products under the breeder's strain or trade name.

Hatchery

Hatchery equipment on one premises operated or controlled by any person for the production of baby poultry.

Infected flock

A flock in which an authorized laboratory has discovered one or more birds infected with a communicable poultry disease for which a program has been established under the Plan.

Midlay

Approximately 2–3 months after a flock begins to lay or after a molted flock is put back into production.

Multiplier breeding flock

A flock that is intended for the production of hatching eggs used for the purpose of producing progeny for commercial egg or meat production or for other non-breeding purposes.

Official State Agency

The State authority recognized by the Department to cooperate in the administration of the Plan.

Official supervision

(1) As applied to Plan programs. The direction, inspection, and critical evaluation by the Official State Agency of compliance with the provisions of the Plan;
(2) As applied to non-Plan but equivalent State poultry improvement programs. The direction, inspection, and critical evaluation by an officer or agency of a State government, of compliance with a publicly announced State poultry improvement program.

Person

A natural person, firm, or corporation.

Plan

The provisions of the National Poultry Improvement Plan contained in this part.

Poultry	Domesticated fowl, including chickens, turkeys, waterfowl, and game birds, except doves and pigeons, which are bred for the primary purpose of producing eggs or meat.
Primary breeding flock	A flock composed of one or more generations that is maintained for the purpose of establishing, continuing, or improving parent lines.
Products	Poultry breeding stock and hatching eggs, baby poultry, and started poultry.
Program	Management, sanitation, testing, and monitoring procedures which, if complied with, will qualify, and maintain qualification for, designation of a flock, products produced from the flock, or a state by an official Plan classification and illustrative design, as described in § 145.10 of this part.
Pullorum disease or pullorum	A disease of poultry caused by *Salmonella pullorum*.
Reactor	A bird that has a positive reaction to a test, required or recommended in Parts 145 or 147 of this chapter, for any poultry disease for which a program has been established under the Plan.
Salmonella	Any bacteria belonging to the genus Salmonella, including the arizona group.
Sanitize	To treat with a product which is registered by the Environmental Protection Agency as germicidal, fungicidal, pseudomonocidal, or tuberculocidal, in accordance with the specifications for use as shown on the label of each product. The Official State Agency, with the concurrence of the Service, shall approve each product or procedure according to its specified usage.
Serial	The total quantity of completed product which has been thoroughly mixed in a single container and identified by a serial number.
Service	The Animal and Plant Health Inspection Service, Veterinary Services, of the Department.
Sexual maturity	The average age at which a species of poultry is biologically capable of reproduction.
Started poultry	Young poultry (chicks, pullets, cockerels, capons, poults, ducklings, goslings, keets, etc.) that have been fed and watered and are less than 6 months of age.
State	Any State, the District of Columbia, or Puerto Rico.
State Inspector	Any person employed or authorized under § 145.11(b) to perform functions under this part.
Stock	A term used to identify the progeny of a specific breeding combination within a species of poultry. These breeding combinations may include pure strains, strain crosses, breed crosses, or combinations thereof.

Strain	Poultry breeding stock bearing a given name produced by a breeder through at least five generations of closed flock breeding.
S. typhimurium* infection or *typhimurium	A disease of poultry caused by *Salmonella typhimurium* or *S. typhimurium* var. copenhagen.
Succeeding flock	A flock brought onto a premises during the 12 months following removal of an infected flock.
Suspect flock	A flock shall be considered, for the purposes of the Plan, to be a suspect flock if any evidence exists that it has been exposed to a communicable poultry disease.
Trade name or number	A name or number compatible with State and Federal laws and regulations applied to a specified stock or product thereof.

§145.2 Administration

(a) The Department cooperates through a Memorandum of Understanding with Official State Agencies in the administration of the Plan.
(b) The administrative procedures and decisions of the Official State Agency are subject to review by the Service. The Official State Agency shall carry out the administration of the Plan within the State according to the applicable provisions of the Plan and the Memorandum of Understanding.
(c) An Official State Agency may accept for participation an affiliated flock located in another State under a mutual understanding and agreement, in writing, between the two Official State Agencies regarding conditions of participation and supervision.
(d) The Official State Agency of any State may, except as limited by § 145.3(d), adopt regulations applicable to the administration of the Plan in such State further defining the provisions of the Plan or establishing higher standards compatible with the Plan.

(Approved by the Office of Management and Budget under control number 0579-0007)

§ 145.3 Participation

(a) Any person producing or dealing in products may participate in the Plan when he has demonstrated, to the satisfaction of the Official State Agency, that his facilities, personnel, and practices are adequate for carrying out the applicable provisions of the Plan, and has signed an agreement with the Official State Agency to comply with the general and the applicable specific provisions of the Plan and any regulations of the Official State Agency under § 145.2. Affiliated flockowners may participate without signing an agreement with the Official State Agency.
(b) Each participant shall comply with the Plan throughout the operating year of the Official State Agency, or until released by such Agency.
(c) A participant in any State shall participate with all of his poultry hatching egg supply flocks and hatchery operations within such State. He shall report to the Official State Agency on VS Form 9-2 (formerly NPIP Form 3B) or through other appropriate means each breeding flock before the birds reach 24 weeks of age. This report will include:
    (1) Name and address of flockowner;
    (2) Flock location and designation;
    (3) Type: Primary or Multiplier;
    (4) Breed, variety, strain, or trade name of stock;
    (5) Source of males;
    (6) Source of females;
    (7) Number of birds in the flock; and
    (8) Intended classification of flock.

(d) No person shall be compelled by the Official State Agency to qualify products for any of the other classifications described in § 145.10 as a condition of qualification for the U.S. Pullorum-Typhoid Clean classification.
(e) Participation in the Plan shall entitle the participant to use the Plan emblem reproduced below:

National Poultry Improvement Plan

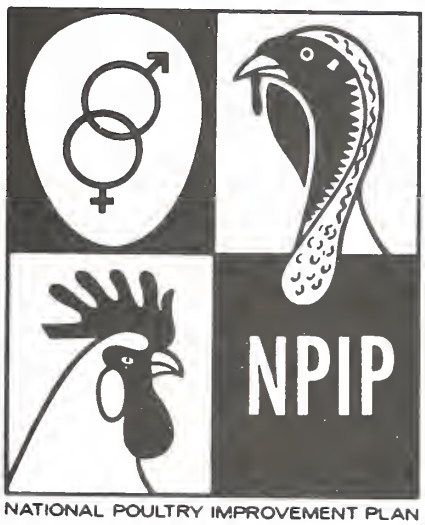

Figure 1

(Approved by the Office of Management and Budget under control number 0579-0007)

§ 145.4 General Provisions for all Participants.

(a) Records of purchases and sales and the identity of products handled shall be maintained in a manner satisfactory to the Official State Agency.
(b) Products, records of sales and purchase of products, and material used to advertise products shall be subject to inspection by the Official State Agency at any time.
(c) Advertising must be in accordance with the Plan, and applicable rules and regulations of the Official State Agency and the Federal Trade Commission. A participant advertising products as being of any official classification may include in his advertising reference to associated or franchised hatcheries only when such hatcheries produce the same kind of products of the same classification.
(d) Except as provided by this paragraph, participants in the Plan may not buy or receive products for any purpose from nonparticipants unless they are part of an equivalent program, as determined by the Official State Agency. Participants in the Plan may buy or receive products from flocks that are neither participants nor

part of an equivalent program, for use in breeding flocks or for experimental purposes, under the following conditions only:

(1) With the permission of the Official State Agency and the concurrence of the Service; and

(2) By segregation of all birds before introduction into the breeding flock. Upon reaching sexual maturity, the segregated birds must be tested and found negative for pullorum-typhoid. The Official State Agency may require a second test at its discretion.

(e) Each participant shall be assigned a permanent approval number by the Service. This number, prefaced by the numerical code of the State, will be the official approval number of the participant and may be used on each certificate, invoice, shipping label, or other document used by the participant in the sale of his products. Each Official State Agency which requires an approval or permit number for out-of-State participants to ship into its State should honor this number. The approval number shall be withdrawn when the participant no longer qualifies for participation in the Plan.

(Approved by the Office of Management and Budget under control number 0579-0057)

§ 145.5 Specific Provisions for Participating Flocks.

(a) Poultry equipment, and poultry houses and the land in the immediate vicinity thereof, shall be kept in sanitary condition as recommended in §§ 147.21 and 147.22(a) and (e) of this chapter. The participating flock, its eggs, and all equipment used in connection with the flock shall be separated from nonparticipating flocks, in a manner acceptable to the Official State Agency.

(b) All flocks shall consist of healthy, normal individuals characteristic of the breed, variety, cross, or other combination which they are stated to represent.

(c) A flock shall be deemed to be a participating flock at any time only if it has qualified for the U.S. Pullorum-Typhoid Clean classification, as prescribed in Subparts B, C, D, or E of this part.

(d) Each bird shall be identified with a sealed and numbered band obtained through or approved by the Official State Agency: *Provided*, That exception may be made at the discretion of the Official State Agency.

§ 145.6 Specific Provisions for Participating Hatcheries.

(a) Hatcheries, including brooder rooms, shall be kept in sanitary condition, acceptable to the Official State Agency. The procedures outlined in §§ 147.22 through 147.25 of this chapter shall be considered as a guide in determining compliance with this provision. The minimum requirements with respect to sanitation shall include the following:
  (1) Incubator walls, floors, and trays shall be kept free from broken eggs and eggshells.
  (2) Tops of incubators and hatchers shall be kept clean (not used for storage).
  (3) Entire hatchery, including sales room, shall be kept in a neat, orderly condition and free from accumulated dust.
  (4) Hatchery residue, such as eggshells, infertile eggs, and dead germs, shall be disposed of promptly and in a manner satisfactory to the Official State Agency.
  (5) Hatchers and hatching trays shall be cleaned and fumigated or disinfected after each hatch, preferably using the procedures outlined in §§ 147.24(b) and 147.25(e) of this chapter. While not mandatory for participation, all eggs set should be fumigated as described in § 147.25 or otherwise sanitized.

(b) A hatchery which keeps started poultry must keep such poultry separated from the incubator room in a manner satisfactory to the Official State Agency.

(c) All baby and started poultry offered for sale under Plan terminology shall be normal and typical of the breed, variety, cross, or other combination represented.

(d) Eggs incubated shall be sound in shell, typical for the breed, variety, strain, or cross thereof and reasonably uniform in shape. Hatching eggs shall be trayed and the baby poultry boxed with a view to uniformity of size.

(e) If a person is responsibly connected with more than one hatchery, all of such hatcheries must participate in the Plan if any of them participate. A person is deemed to be responsibly connected with a hatchery if he or she is a partner, officer, director, holder, owner of 10 percent or more of the voting stock, or an employee in a managerial or executive capacity.

§ 145.7 Specific Provisions for Participating Dealers.

Dealers in poultry breeding stock, hatching eggs, or baby or started poultry shall comply with all provisions in this part which apply to their operations.

§ 145.8 Terminology and Classification; General.

(a) The official classification terms defined in §§ 145.9 and 145.10 and the various designs illustrative of the official classifications reproduced in § 145.10 may be used only by participants and to describe products that have met all the specific requirements of such classifications.

(b) Products produced under the Plan shall lose their identity under Plan terminology when they are purchased for resale by or consigned to nonparticipants.

(c) Participating flocks, their eggs, and the baby and started poultry produced from them may be designated by their strain or trade name. When a breeder's trade name or strain designation is used, the participant shall be able by records to substantiate that the products so designated are from flocks that are composed of either birds hatched from eggs produced under the direct supervision of the breeder of such strain, or stock multiplied by persons designated and so reported by the breeder to each Official State Agency concerned.

§ 145.9 Terminology and Classification; Hatcheries and Dealers.

Participating hatcheries and dealers shall be designated as "National Plan Hatchery" and "National Plan Dealer", respectively. All Official State Agencies shall be notified by the Service of additions, withdrawals, and changes in classification.

§ 145.10 Terminology and Classification; Flocks, Products, and States.

Participating flocks, products produced from them, and States which have met the respective requirements specified in Part 145 Subpart B, C, D, or E may be designated by the following terms or illustrative designs:

(a) U.S. Approved. (See § 145.53(a).)

(a)

Figure 2

(b) U.S. Pullorum-Typhoid Clean. (See § 145.23(b), § 145.33(b), § 145.43(b), and § 145.53(b).)

(b)

Figure 3

(c) U.S. *M. gallisepticum* Clean. (See § 145.23(c), § 145.23(f), § 145.33(c), § 145.33(f), § 145.43(c), and § 145.53(c).)

(c)

Figure 4

(d) U.S. Sanitation Monitored. (See § 145.33(d).)

(d)

Figure 5

(e) U.S. *M. synoviae* Clean. (See § 145.23(e), § 145.23(g), § 145.33(e), § 145.33(g), and § 145.43(e).)

(e)

Figure 6

(f) U.S. *M. meleagridis* Clean. (See § 145.43(d).)

(f)

Figure 7.

(g) U.S. Pullorum-Typhoid Clean State. (See § 145.24(a), § 145.34(a), § 145.44(a), and § 145.54(a).)

(g)

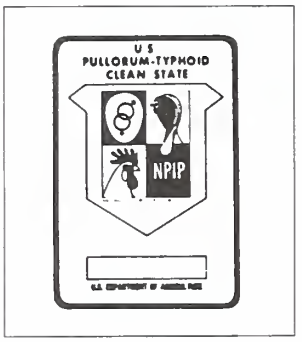

Figure 8

(h) U.S. Pullorum-Typhoid Clean State, Turkeys. (See § 145.44(b).)

(h)

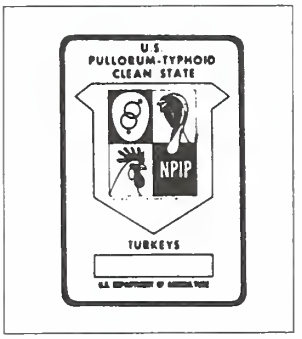

Figure 9

(i) U.S. *M. gallisepticum* Clean State, Turkeys. (See § 145.44(c).)

(i)

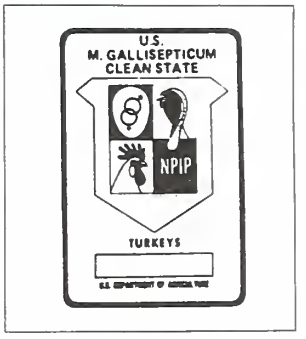

Figure 10

(j) U.S. *M. gallisepticum* Clean State, Meat-Type Chickens. (See § 145.34(b).)

(j)

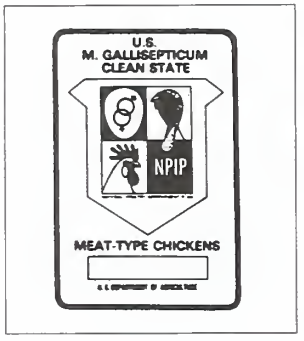

Figure 11

(k) U.S. Sanitation Monitored, Turkeys. (See § 145.43(f).)

(k)

Figure 12

(l) U.S. *S. enteritidis* Monitored. (See § 145.23 (d).)

(l)

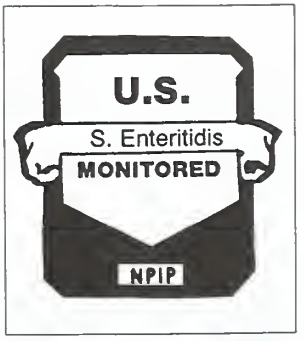

Figure 13

§ 145.11 Supervision.

(a) The Official State Agency may designate qualified persons as Authorized Agents to do the sample collecting and blood testing provided for in § 145.14 and the selecting required for the U.S. Approved classification provided for in § 145.53(a).
(b) The Official State Agency shall employ or authorize qualified persons as State Inspectors to perform or supervise the performance of the selecting and testing of participating flocks, and to perform the official inspections necessary to verify compliance with the requirements of the Plan.
(c) Authorities issued under the provisions of this section shall be subject to cancellation by the Official State Agency on the grounds of incompetence or failure to comply with the provisions of the Plan or regulations of the Official State Agency. Such actions shall not be taken until a thorough investigation has been made by the Official State Agency and the authorized person has been given notice of the proposed action and the basis therefor and an opportunity to present his views.

§ 145.12 Inspections.

(a) Each participating hatchery shall be inspected a sufficient number of times each year to satisfy the Official State Agency that the operations of the hatchery are in compliance with the provisions of the Plan.
(b) The records of all flocks maintained primarily for production of hatching eggs shall be examined annually by a State Inspector. Records shall include VS Form 9-2, "Flock Selecting and Testing Report"; VS Form 9-3, "Report of Sales of Hatching Eggs, Chicks, and Poults"; set and hatch records; egg receipts; and egg/chick orders or invoices. Records shall be maintained for 3 years. On-site inspections of flocks and premises will be conducted if the State Inspector determines that a breach of sanitation, blood testing, or other provisions has occurred for Plan programs for which the flocks have or are being qualified.

§ 145.13 Debarment from participation.

Participants in the Plan, who after investigation by the Official State Agency or its representative, are notified of their apparent noncompliance with the Plan provisions or regulations of the Official State Agency, shall be afforded a reasonable time, as specified by the Official State Agency, within which to demonstrate or achieve compliance. If compliance is not demonstrated or achieved within the specified time, the Official State Agency may debar the participant from further participation in the Plan for such period, or indefinitely, as the Agency may deem appropriate. The debarred participant shall be afforded notice of the bases for the debarment and opportunity to present his views with respect to the debarment in accordance with

procedures adopted by the Official State Agency. The Official State Agency shall thereupon decide whether the debarment order shall continue in effect. Such decision shall be final unless the debarred participant, within 30 days after the issuance of the debarment order, requests the Administrator to determine the eligibility of the debarred participant for participation in the Plan. In such event the Administrator shall determine the matter de novo in accordance with §§ 50.21 through 50.28-14 and §§ 50.30 through 50.33 of the rules of practice in 7 CFR Part 50, which are hereby made applicable to proceedings before the Administrator under this section. The definitions in 7 CFR 50.2(e), (g), (h), and (l) and the following definitions shall apply with respect to terms used in such rules of practice:

(a) "Administrator" means the Administrator, Animal and Plant Health Inspection Service of the U.S. Department of Agriculture or any officer or employee to whom authority has heretofore been delegated or to whom authority may hereafter be delegated to act in his stead.

§ 145.14 Blood testing.

Poultry must be more than 4 months of age when blood tested for an official classification: *Provided,* That turkey candidates may be blood tested at more than 12 weeks of age under Subpart D, while game birds may be blood tested under Subpart E when more than 4 months of age or upon reaching sexual maturity, whichever comes first. Blood samples for official tests shall be drawn by an Authorized Agent or State Inspector and tested by an authorized laboratory, except that the stained antigen, rapid whole-blood test for pullorum-typhoid may be conducted by an Authorized Agent or State Inspector. For Plan programs in which a representative sample may be tested in lieu of an entire flock, the minimum number tested shall be 30 birds per house, with at least 1 bird taken from each pen and unit in the house. All birds must be tested in houses containing fewer than 30 birds.

(a) For Pullorum-Typhoid

(1) The official blood tests for pullorum-typhoid shall be the standard tube agglutination test, the microagglutination test, the enzyme-labeled immunosorbent assay test (ELISA), or the rapid serum test for all poultry; and the stained antigen, rapid whole-blood test for all poultry except turkeys. The procedures for conducting official blood tests are set forth in §§ 147.1, 147.2, 147.3, and 147.5 of this chapter and referenced in footnote 3 of this section or in literature provided by the producer. Only antigens approved by the Department and of the polyvalent type shall be used for the rapid whole-blood and tube agglutination tests. Each serial of tube antigen shall be submitted by the antigen producer to the Department for approval upon manufacture and once a year thereafter as long as antigen from that serial continues to be made available for use. All microtest antigens and enzyme-labeled immunosorbent assay reagents shall also be approved by the Department.[1]

[1] The criteria and procedures for Department approval of antigens and reagents may be obtained from Veterinary Biologics, BBEP, APHIS, USDA, Federal Building, 6505 Belcrest Road, Hyattsville, MD 20782.

(2) [Reserved]
(3) There shall be an interval of at least 21 days between any official blood test and any previous test with pullorum-typhoid antigen.
(4) [Reserved]
(5) The official blood test shall include the testing of a sample of blood from each bird in the flock: *Provided*, That under specified conditions (see applicable provisions of §§ 145.23, 145.33, 145.43 and 145.53) the testing of a portion or sample of the birds may be used in lieu of testing each bird.
(6) When reactors are found from any flock, or S. pullorum or S. gallinarum organisms are isolated by an authorized laboratory from baby poultry, or from fluff samples produced by hatching eggs, the infected flock shall qualify for participation in the Plan with two consecutive negative results to an official blood test named in paragraph (a)(1) of this section. A succeeding flock must be qualified for participation in the Plan's pullorum-typhoid program with a negative result to an official blood test named in paragraph (a)(1) of this section. Testing to qualify flocks for Plan participation must include the testing of all birds in infected and succeeding flocks for a twelve month period, and shall be performed or physically supervised by a State Inspector: *Provided,* That at the discretion of the Official State Agency, a sample of at least 500 birds, rather than all birds in the flock, may be tested by the State Inspector if it is agreed upon by the Official State Agency, the flockowner, and the Administrator. If the State Inspector determines that a primary breeding flock has been exposed to *S. pullorum* or *S. gallinarum*,[2] the Official State Agency may require:
    (i) The taking of blood samples—performed by or in the presence of a State Inspector—from all birds on premises exposed to birds, equipment, supplies, or personnel from the primary breeding flock during the period when the State Inspector determined that exposure to *S. pullorum* or *S. gallinarum* occurred.[2]
    (ii) The banding of all birds on these premises—performed or physically supervised by a State Inspector—in order to identify any bird that tests positive; and
    (iii) The testing of blood samples at an authorized laboratory using an official blood test named in paragraph (a)(1) of this section.
(7) All domesticated fowl, except waterfowl, on the farm of the participant shall either be properly tested to meet the same standards as the participating flock or these birds and their eggs shall be separated from the participating flock and its eggs.
(8) All tests for pullorum-typhoid in flocks participating in or candidates for participation in the Plan shall be reported to the Official State Agency within 10 days following the completion of such tests. All reactors shall be considered in determining the classification of the flock.
(9) Poultry from flocks undergoing qualification testing for participation in the Plan, that have a positive reaction to an official blood test named in paragraph (a)(1) of this section, shall be evaluated for pullorum-typhoid infection. The Official State Agency shall select one or more of the following procedures to be used in each

[2]In making determinations of exposure, the State Inspector shall evaluate both evidence proving that exposure occurred and circumstances indicating a high probability of contacts with: infected wild birds; contaminated feed or waste; or birds, equipment, supplies, or persons from or exposed to flocks infected with *S. pullorum* or *S. gallinarum*.

circumstance, based on a cost-benefit analysis involving evaluation of such factors as: the value of the reactors and flocks at risk; the necessity for preserving birds from scarce genetic lines; the need for a quick determination of disease existence; and the cost for each retesting option versus the total availability of funds (when the State provides retesting subsidies):

(i) Reactors shall be submitted to an authorized laboratory for bacteriological examination. If there are more than 4 reactors in a flock, a minimum of 4 reactors shall be submitted to the authorized laboratory; if the flock has 4 or fewer reactors, all of the reactors must be submitted. The approved procedure for bacteriological examination is set forth in § 147.11 of this chapter. When reactors are submitted to the authorized laboratory within 10 days from the date of reading an official blood test named in paragraph (a)(1) of this section, and the bacteriologicial examination fails to demonstrate pullorum-typhoid infection, the Official State Agency shall presume that the flock has no pullorum-typhoid reactors.

(ii) The serum specimen that produced the positive reaction shall be retested at an authorized laboratory in accordance with procedures set forth in § 147.1 of this chapter for the standard tube agglutination test, or in § 147.5 of this chapter for the microagglutination test for pullorum-typhoid. If the reaction to this retest is positive in dilutions of 1:50 or greater for the standard tube agglutination test, or 1:40 or greater for the microagglutination test, additional examination of the bird and flock will be performed in accordance with paragraph (a)(9)(i) or (a)(9)(iii) of this section.

(iii) The reactors shall be retested within 30 days using an official blood test named in paragraph (a)(1) of this section. If this retest is positive, additional examination of the reactors and flock will be performed in accordance with paragraph (a)(9)(i) of this section. During this 30-day period, the flock must be maintained under a security system, specified or approved by the Official State Agency, that will prevent physical contact with other birds and assure that personnel, equipment, and supplies that could be a source of pullorum-typhoid spread are sanitized.

(10) Any drug, for which there is scientific evidence of masking the test reaction or hindering the bacteriological recovery of Salmonella organisms, shall not be fed or administered to poultry within 3 weeks prior to a test or bacteriological examination upon which a Salmonella classification is based.

(11) When suitable evidence, as determined by the Official State Agency or the State Animal Disease Control Official, indicates that baby or started poultry produced by participating hatcheries are infected with organisms for which the parent flock received an official control classification and this evidence indicates that the infection was transmitted from the parent flock, the Official State Agency may, at its discretion, require additional testing of the flock involved. If infection is found in the parent flock, its classification shall be suspended until the flock is requalified under the requirements for the classification. Furthermore, the Official State Agency may require that the hatching eggs from such flocks be removed from the incubator and destroyed prior to hatching. When Salmonella organisms are

isolated from a specimen which originated in a participating hatchery, the Official State Agency shall attempt to locate the source of the infection. The results of the investigation and the action taken to eliminate the infection shall be reported by the Official State Agency to the Service.

(b) For *M. gallisepticum* and *M. synoviae*

(1) The official blood tests for *M. gallisepticum* and *M. synoviae* shall be the serum plate agglutination test, the tube agglutination test, the hemagglutination inhibition (HI) test, the microhemagglutination inhibition test, the enzyme-labeled immunosorbent assay (ELISA) test[3] or a combination of two or more of these tests. The HI test, the microhemagglutination inhibition test, and the ELISA test shall be used to confirm the positive results of other serological tests. HI titers of 1:40 or less may be interpreted as equivocal, and final judgment may be based on further samplings and/or culture of reactors.

(2) The tests shall be conducted using *M. gallisepticum* or *M. synoviae* antigens approved by the Department or the Official State Agency and shall be performed in accordance with the recommendations of the producer of the antigen.

(3) When reactors to the test for which the flock was tested are submitted to a laboratory as prescribed by the Official State Agency, the criteria found in § 147.6 shall be used in determining the final status of the flock.

(4) Any drug, for which there is scientific evidence of masking the test reaction or hindering the bacteriological recovery of mycoplasma organisms, shall not be fed or administered to poultry within three weeks prior to a test or bacteriological examination upon which a Mycoplasma classification is based.

(c) For *M. meleagridis*

The official blood tests for *M. meleagridis* are specified in § 145.43(d)(2).

(Approved by the Office of Management and Budget under control number 0579-0007)

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[3]Procedures for the enzyme-labeled immunosorbent assay (ELISA) test are set forth in the following publications:
A.A. Ansari, R.F. Taylor, T.S. Chang, "Application of Enzyme-Linked Immunosorbent Assay for Detecting Antibody to Mycoplasma Gallisepticum Infections in Poultry," Avian Diseases, Vol. 27, No. 1, pp. 21–35, January–March 1983; and
H.M. Opitz, J.B. Duplessis, and M.J. Cyr, "Indirect Micro-Enzyme-Linked Immunosorbent Assay for the Detection of Antibodies to *Mycoplasma synoviae* and *M. gallisepticum*," Avian Diseases, Vol. 27, No. 3, pp. 773–786, July–September 1983; and
H.B. Ortmayer and R. Yamamoto, "*Mycoplasma meleagridis* Antibody Detection by Enzyme-Linked Immunosorbent Assay (ELISA)," Proceedings, 30th Western Poultry Disease Conference, pp. 63–66, March 1981.

Subpart B—Special Provisions for Egg-Type Chicken Breeding Flocks and Products

§ 145.21 Definitions.

Except where the context otherwise requires, for the purposes of this subpart the following terms shall be construed, respectively, to mean:

Chicks Newly hatched chickens which have not been fed or watered.

Egg-type chicken breeding flocks Flocks that are composed of stock that has been developed for egg production and are maintained for the principal purpose of producing chicks for the ultimate production of eggs for human consumption.

Started chickens Young chickens (chicks, pullets, cockerels, capons) which have been fed and watered and are less than 6 months of age.

§ 145.22 Participation.

Participating flocks of egg-type chickens, and the eggs and chicks produced from them, shall comply with the applicable general provisions of Subpart A of this part and the special provisions of this Subpart B.

(a) The minimum weight of hatching eggs sold shall be 1 11/12 ounces each, except as otherwise specified by the purchaser of the eggs.
(b) Mediterranean breed eggs shall be reasonably free from tints.
(c) Started chickens shall lose their identity under Plan terminology when not maintained by Plan participants under the conditions prescribed in § 145.5(a).
(d) Hatching eggs produced by primary breeding flocks shall be fumigated (see § 147.25 of this chapter) or otherwise sanitized.

§ 145.23 Terminology and Classification; Flocks and Products.

Participating flocks, and the eggs and chicks produced from them, which have met the respective requirements specified in this section may be designated by the following terms and the corresponding designs illustrated in § 145.10:

(a) [Reserved]

(b) U.S. Pullorum-Typhoid Clean A flock in which freedom from pullorum and typhoid has been demonstrated to the Official State Agency under the criteria in one of the following paragraphs (b)(1) through (5) of this section: *Provided*, That a flock qualifying by means of a blood test shall be tested within the past 12 months, except that the retesting of a participating flock which is retained for more than 12 months shall be at the discretion of the Official State Agency with the concurrence of the Service. (See § 145.14 relating to the official blood test where applicable.)

(1) It has been officially blood tested with no reactors.

(2) It is a multiplier breeding flock, or a breeding flock composed of progeny of a primary breeding flock which is intended solely for the production of multiplier breeding flocks, and meets the following specifications as determined by the Official State Agency and the Service:

(i) The flock is located in a State where all persons performing poultry disease diagnostic services within the State are required to report to the Official State Agency within 48 hours the source of all poultry specimens from which *S. pullorum* or *S. gallinarum* is isolated;

(ii) The flock is composed entirely of birds that originated from U.S. Pullorum-Typhoid Clean breeding flocks or from flocks that met equivalent requirements under official supervision; and

(iii) The flock is located on a premises where either no poultry or a flock not classified as U.S. Pullorum-Typhoid Clean were located the previous year: *Provided*, That an Authorized Agent must blood test up to 300 birds per flock, as described in § 145.14, if the Official State Agency determines that the flock has been exposed to pullorum-typhoid. In making determinations of exposure and setting the number of birds to be blood tested, the Official State Agency shall evaluate the results of any blood tests, described in § 145.14(a)(1) of this part, that were performed on an unclassified flock located on the premises during the previous year; the origins of the unclassified flock; and the probability of contacts between the flock for which qualification is being sought and (a) infected wild birds, (b) contaminated feed or waste, or (c) birds, equipment, supplies, or personnel from flocks infected with pullorum-typhoid.

(3) It is a multiplier breeding flock that originated from U.S. Pullorum-Typhoid Clean breeding flocks or from flocks that met equivalent requirements under official supervision, and is located in a State in which it has been determined by the Service that:

(i) All hatcheries, except turkey hatcheries, within the State are qualified as "National Plan Hatcheries" or have met equivalent requirements for pullorum-typhoid control under official supervision;

(ii) All hatchery supply flocks, except turkey flocks, within the State, are qualified as U.S. Pullorum-Typhoid Clean or have met equivalent requirements for pullorum-typhoid control under official supervision: *Provided*, That if other domesticated fowl, except waterfowl, are maintained on the same premises as the participating flock, freedom from pullorum-typhoid infection shall be demonstrated by an official blood test of each of these fowl;

(iii) All shipments of products other than U.S. Pullorum-Typhoid Clean, or equivalent, into the State are prohibited;

(iv) All persons performing poultry disease diagnostic services within the State are required to report to the Official State Agency within 48 hours the source of all poultry specimens from which *S. pullorum* or *S. gallinarum* is isolated;

(v) All reports of any disease outbreak involving a disease covered under the Plan are promptly followed by an investigation by the Official State Agency to determine the origin of the infection: *Provided,* That if the origin of the

infection involves another State, or if there is exposure to poultry in another State from the infected flock, then the National Poultry Improvement Plan will conduct an investigation;

(vi) All flocks found to be infected with pullorum or typhoid are quarantined until marketed or destroyed under the supervision of the Official State Agency, or until subsequently blood tested, following the procedure for reacting flocks as contained in § 145.14(a)(5), and all birds fail to demonstrate pullorum or typhoid infection;

(vii) All poultry, including exhibition, exotic, and game birds, but excluding waterfowl, going to public exhibition shall come from U.S. Pullorum-Typhoid Clean or equivalent flocks, or have had a negative pullorum- typhoid test within 90 days of going to public exhibition;

(viii) Discontinuation of any of the conditions or procedures described in paragraphs (b)(3)(i), (ii), (iii), (iv), (v), (vi), and (vii) of this section, or the occurrence of repeated outbreaks of pullorum or typhoid in poultry breeding flocks, other than turkey flocks, within or originating within the State shall be grounds for the Service to revoke its determination that such conditions and procedures have been met or complied with. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity to present its views.

(4) It is a multiplier breeding flock located in a State which has been determined by the Service to be in compliance with the provisions of (b)(3) of this section, and in which pullorum disease or fowl typhoid is not known to exist nor to have existed in hatchery supply flocks, other than turkey, waterfowl, exhibition poultry, and game bird supply flocks, within the State during the preceding 12 months.

(5) It is a primary breeding flock located in a State determined to be in compliance with the provisions of paragraph (b)(4) of this section, and in which a sample of 300 birds from flocks of more than 300, and each bird in flocks of 300 or less, has been officially tested for pullorum-typhoid with no reactors: *Provided*, That a bacteriological examination monitoring program acceptable to the Official State Agency and approved by the Service may be used in lieu of blood testing.

(c) U.S. *M. gallisepticum* Clean

(1) A flock maintained in compliance with the provisions of § 147.26 of this chapter and in which freedom from *M. gallisepticum* has been demonstrated under the criteria specified in paragraph (c)(1)(i) or (ii) of this section.

(i) It is a flock in which all birds or a sample of at least 300 birds has been tested for *M. gallisepticum* as provided in § 145.14(b) when more than 4 months of age: *Provided*, That to retain this classification, a minimum of 150 birds shall be tested at intervals of not more than 90 days: *And provided further*, That a sample comprised of less than 150 birds may be tested at any one time, with the approval of the Official State Agency and the concurrence of the Service, provided that a minimum of 150 birds is tested within each 90-day period; or

(ii) It is a multiplier breeding flock which originated as U.S. *M. gallisepticum* Clean chicks from primary breeding flocks and from which a sample comprised of a minimum of 150 birds per flock has been tested for *M. gallisepticum* as

provided in § 145.14(b) when more than 4 months of age: *Provided*, That to retain this classification, the flock shall be subjected to one of the following procedures:

(A) At intervals of not more than 90 days, a sample of 75 birds, with a minimum of 30 birds per pen, whichever is greater, shall be tested; or

(B) At intervals of not more than 30 days, a sample of 25 cull chicks produced from the flock shall be subjected to laboratory procedures acceptable to the Official State Agency and approved by the Service, for the detection and recovery of *M. gallisepticum*; or

(C) At intervals of not more than 30 days, egg yolk testing shall be conducted in accordance with § 147.8.

(2) A participant handling U.S. *M. gallisepticum* Clean products shall keep these products separate from other products in a manner satisfactory to the Official State Agency: *Provided*, That U.S. *M. gallisepticum* Clean chicks from primary breeding flocks shall be produced in incubators and hatchers in which only eggs from flocks qualified under paragraph (c) (1) (i) of this section are set.

(3) U.S. *M. gallisepticum* Clean chicks shall be boxed in clean boxes and delivered in trucks that have been cleaned and disinfected as described in § 147.24 (a) of this chapter.

(d) U.S. *S. enteritidis* Monitored

This program is intended to be the basis from which the breeding-hatching industry may conduct a program for the prevention and control of Salmonellosis. It is intended to reduce the incidence of Salmonella organisms in hatching eggs and chicks through an effective and practical sanitation program at the breeder farm and in the hatchery. This will afford other segments of the poultry industry an opportunity to reduce the incidence of Salmonella in their products.

(1) A flock and the hatching eggs and chicks produced from it which have met the following requirements as determined by the Official State Agency:

(i) The flock originated from a U.S. Sanitation Monitored flock, or meconium from the chick boxes and a sample of chicks that died within 7 days after hatching are examined bacteriologically for salmonella at an authorized laboratory. Cultures from positive samples shall be serotyped.

(ii) All feed fed to the flock shall meet the following requirements:

(A) Pelletized feed shall contain either no animal protein or only animal protein products produced under the Animal Protein Products Industry (APPI) Salmonella Education/Reduction Program[1]. The protein products must have a minimum moisture content of 14.5 percent and must have been heated throughout to a minimum temperature of 190 °F, or above, or to a minimum temperature of 165 °F. for at least 20 minutes, or to a minimum temperature of 184 °F. under 70 lbs. pressure during the manufacturing process.

(B) Mash feed shall contain either no animal protein or only animal protein products supplement manufactured in pellet form and crumbled.

[1]Documents concerning the APPI/Salmonella Education Reduction Program may be obtained from Mr. A. R. Rhorer; Sheep, Goat, Equine, and Poultry Diseases Staff; VS, APHIS, USDA; Room 205 Presidential Building; 6525 Belcrest Road; Hyattsville, Maryland 20782.

(iii) Feed shall be stored and transported in such a manner as to prevent possible contamination;

(iv) The flock is maintained in compliance with §§ 147.21, 147.24(a), and 147.26 of this chapter;

(v) Environmental samples shall be collected from the flock by an Authorized Agent, as described in § 147.12 of this chapter, when the flock is 2 to 4 weeks of age. The Authorized Agent shall also collect samples every 30 days after the first sample has been collected. The samples shall be examined bacteriologically for group D salmonella at an authorized laboratory. Cultures from positive samples shall be serotyped.

(vi) A federally licensed *Salmonella enteritidis* bacterin may be used in multiplier breeding flocks that are negative for *Salmonella enteritidis* upon bacteriological examination as described in paragraph (d)(1)(v) of this section: *Provided,* That a sample of 350 birds, which will be banded for identification, shall remain unvaccinated until the flock reaches at least 4 months of age. Following negative serological and bacteriological examinations as described in paragraph (d)(1)(vii) of this section, the banded, non-vaccinated birds shall be vaccinated.

(vii) Blood samples from 300 non-vaccinated birds as described in paragraph (d)(1)(vi) of this section shall be officially tested with pullorum-typhoid antigen when the flock is a minimum of more than 4 months of age. All birds with positive or inconclusive reactions, up to a maximum of 25 birds, shall be submitted to an authorized laboratory and examined for the presence of group D salmonella, as described in § 147.11 of this chapter. Cultures from positive samples shall be serotyped.

(viii) Hatching eggs are collected as quickly as possible and are handled as described in § 147.22 of this chapter and are sanitized or fumigated (see § 147.25 of this chapter).

(ix) Hatching eggs produced by the flock are incubated in a hatchery that is in compliance with the recommendations in §§ 147.23 and 147.24(b) of this chapter, and sanitized either by a procedure approved by the Official State Agency or fumigated (see § 147.25 of this chapter).

(2) A flock shall not be eligible for this classification if *Salmonella enteritidis* ser enteritidis (SE) is isolated from a specimen taken from a bird in the flock. Isolation of SE from an environmental or other specimen as described in section (d)(1)(v) of this paragraph will require bacteriological examination for SE in an authorized laboratory, as described in § 147.11(a) of this chapter, of a random sample of 60 live birds from a flock of 5,000 birds or more, or 30 live birds from a flock with fewer than 5,000 birds. If only one specimen is found positive for SE, the participant may request bacteriological examination of a second sample, equal in size to the first sample, from the flock. If no SE is recovered from any of the specimens in the second sample, the flock will be eligible for the classification.

(3) A flock shall be eligible for this classification if *Salmonella enteritidis* (*S. enteritidis* ser Enteritidis) is isolated from an environmental sample collected from the flock in accordance with paragraph (d)(v) of this section: *Provided*, That testing is conducted in accordance with paragraph (d)(1)(vi) of this section each 30 days and no positive samples are found.

(4) In order for a hatchery to sell products of this classification, all products handled shall meet the requirements of the classification.
(5) This classification may be revoked by the Official State Agency if the participant fails to follow recommended corrective measures.

(e) U.S. *M. synoviae* Clean

(1) A flock maintained in compliance with the provisions of § 147.26 and in which freedom from *M. synoviae* has been demonstrated under the criteria specified in paragraph (e)(1)(i) or (ii) of this section.
  (i) It is a flock in which a minimum of 300 birds has been tested for *M. synoviae* as provided in § 145.14(b) when more than 4 months of age: *Provided*, That to retain this classification, a sample of at least 150 birds shall be tested at intervals of not more than 90 days: *And provided further*, That a sample comprised of less than 150 birds may be tested at any one time, with the approval of the Official State Agency and the concurrence of the Service, provided that a minimum of 150 birds is tested within each 90-day period; or
  (ii) It is a multiplier breeding flock which originated as U.S. *M. Synoviae* Clean chicks from primary breeding flocks and from which a sample comprised of a minimum of 75 birds has been tested for *M. synoviae* as provided in § 145.14(b) when more than 4 months of age: *Provided*, That to retain this classification, the flock shall be subjected to one of the following procedures:
    (a) At intervals of not more than 90 days, a sample of 50 birds shall be tested: *Provided*, That a sample of less than 50 birds may be tested at any one time, provided that a minimum of 30 birds per flock with a minimum of 15 birds per pen, whichever is greater, is tested each time and a total of at least 50 birds is tested within each 90-day period; or
    (b) At intervals of not more than 30 days, egg yolk testing shall be conducted in accordance with § 147.8.
(2) A participant handling U.S. *M. Synoviae* Clean products shall keep these products separate from other products in a manner satisfactory to the Official State Agency: *Provided*, That U.S. *M. synoviae* Clean chicks from primary breeding flocks shall be produced in incubators and hatchers in which only eggs from flocks qualified under paragraph (e)(1)(i) or (ii) of this section are set.
(3) U.S. *M. Synoviae* Clean chicks shall be boxed in clean boxes and delivered in trucks that have been cleaned and disinfected as described in § 147.24(a).

(f) U.S. *M. gallisepticum* Clean Started Poultry

(1) A flock which originated from U.S. *M. Gallisepticum* Clean breeding flocks and was hatched in a hatchery approved by the Official State Agency for the production of U.S. *M. Gallisepticum* Clean chicks.
(2) All other poultry on the premises of the candidate flock must originate from U.S. *M. Gallisepticum* Clean sources.
(3) The flock is maintained in compliance with the provisions of § 147.26.
(4) The flock's freedom from *M. gallisepticum* is demonstrated by a negative blood test, as provided in § 145.14(b), of a sample of 75 birds, with a minimum of 50 birds per poultry house, between 15–20 days prior to the flock being moved to laying quarters.
(5) Started poultry shall be delivered to and from the farm premises in crates and vehicles which have been cleaned and disinfected as described in § 145.24(a) of this chapter.

(g) U.S. *M. Synoviae* Clean Started Poultry

(1) A flock which originated from U.S. *M. Synoviae* Clean breeding flocks and was hatched in a hatchery approved by the Official State Agency for production of U.S. *M. Synoviae* Clean chicks.
(2) All other poultry on the premises of the candidate flock must originate from U.S. *M. Synoviae* Clean sources.
(3) The flock is maintained in compliance with the provisions of § 147.26.
(4) The flocks' freedom from *M. synoviae* is demonstrated by a negative blood test, as provided in § 145.14(b), of a sample of 75 birds, with a minimum of 50 birds per poultry house, between 15–20 days prior to the flock being moved to laying quarters.
(5) Started poultry shall be delivered to and from the farm premises in crates and vehicles which have been cleaned and disinfected as described in § 147.24(a) of this chapter.

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§ 145.24 Terminology and Classification; States.

(a) U.S. Pullorum-Typhoid Clean State

(1) A State will be declared a U.S. Pullorum-Typhoid Clean State when it has been determined by the Service that:
  (i) The State is in compliance with the provisions contained in § 145.23(b)(3)(i) through (vii), § 145.33(b)(3)(i) through (vii), § 145.43(b)(3)(i) through (vi), and § 145.53(b)(3)(i) through (vii).
  (ii) No pullorum disease or fowl typhoid is known to exist nor to have existed in hatchery supply flocks within the State during the preceding 12 months: *Provided,* That pullorum disease or fowl typhoid found within the preceding 24 months in waterfowl, exhibition poultry, and game bird breeding flocks will not prevent a State, which is otherwise eligible, from qualifying.
(2) Discontinuation of any of the conditions described in paragraph (a) (1) (i) of this section, or repeated outbreaks of pullorum or typhoid occur in hatchery supply flocks described in paragraph (a) (1) (ii) of this section, or if an infection spreads from the originating premises, the Service shall have grounds to revoke its determination that the State is entitled to this classification. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity for a hearing.

Subpart C—Special Provisions for Meat-Type Chicken Breeding Flocks and Products

§ 145.31 Definitions.

Except where the context otherwise requires, for the purposes of this subpart the following terms shall be construed, respectively, to mean:

Chicks Newly hatched chickens which have not been fed or watered.

Meat-type chicken breeding flocks Flocks that are composed of stock that has been developed for meat production and are maintained for the principal purpose of producing chicks for the ultimate production of meat.

Started chickens Young chickens (chicks, pullets, cockerels, capons) which have been fed and watered and are less than 6 months of age.

§ 145.32 Participation.

Participating flocks of meat type chickens, and the eggs and chicks produced from them, shall comply with the applicable general provisions of Subpart A of this part and the special provisions of this Subpart C.

(a) The minimum weight of hatching eggs sold shall be 1 10/12 ounces each, except as otherwise specified by the purchaser of the eggs.

(b) Started chickens shall lose their identity under Plan terminology when not maintained by Plan participants under the conditions prescribed in § 145.5(a).

(c) Hatching eggs produced by primary breeding flocks shall be fumigated (see § 147.25 of this chapter) or otherwise sanitized.

§ 145.33 Terminology and Classification; Flocks and Products.

Participating flocks, and the eggs and chicks produced from them, which have met the respective requirements specified in this section may be designated by the following terms and the corresponding designs illustrated in § 145.10:

(a) [Reserved]

(b) U.S. Pullorum-Typhoid Clean A flock in which freedom from pullorum and typhoid has been demonstrated to the Official State Agency under the criteria in one of paragraphs (b)(1) through (5) of this section: *Provided*, That a flock qualifying by means of a blood test shall be tested within the past 12 months, except that the retesting of a participating flock which is retained for more than 12 months shall be at the discretion of the Official State Agency with the concurrence of the Service. (See § 145.14 relating to the official blood test where applicable.)

(1) It has been officially blood tested with no reactors.

(2) It is a multiplier breeding flock, or a breeding flock composed of progeny of a primary breeding flock which is intended solely for the production of multiplier breeding flocks, and meets the following specifications as determined by the Official State Agency and the Service:

(i) The flock is located in a State where all persons performing poultry disease diagnostic services within the State are required to report to the Official State Agency within 48 hours the source of all poultry specimens from which *S. pullorum* or *S. gallinarum* is isolated;

(ii) The flock is composed entirely of birds that originated from U.S. Pullorum-Typhoid Clean breeding flocks or from flocks that met equivalent requirements under official supervision; and

(iii) The flock is located on a premises where either no poultry or a flock not classified as U.S. Pullorum-Typhoid Clean were located the previous year; *Provided*, That an Authorized Agent must blood test up to 300 birds per flock, as described in § 145.14, if the Official State Agency determines that the flock has been exposed to pullorum-typhoid. In making determinations of exposure and setting the number of birds to be blood tested, the Official State Agency shall evaluate the results of any blood tests, described in § 145.14(a)(1) of this part, that were performed on an unclassified flock located on the premises during the previous year; the origins of the unclassified flock; and the probability of contacts between the flock for which qualification is being sought and (a) infected wild birds, (b) contaminated feed or waste, or (c) birds, equipment, supplies, or personnel from flocks infected with pullorum-typhoid.

(3) It is a multiplier breeding flock that originated from U.S. Pullorum-Typhoid Clean breeding flocks or from flocks that met equivalent requirements under official supervision, and is located in a State in which it has been determined by the Service that:

(i) All hatcheries, except turkey hatcheries, within the State are qualified as "National Plan Hatcheries" or have met equivalent requirements for pullorum-typhoid control under official supervision;

(ii) All hatchery supply flocks, except turkey flocks, within the State, are qualified as U.S. Pullorum-Typhoid Clean or have met equivalent requirements for pullorum-typhoid control under official supervision: *Provided*, That if other domesticated fowl, except waterfowl, are maintained on the same premises as the participating flock, freedom from pullorum-typhoid infection shall be demonstrated by an official blood test of each of these fowl;

(iii) All shipments of products other than U.S. Pullorum-Typhoid Clean, or equivalent, into the State are prohibited;

(iv) All persons performing poultry disease diagnostic services within the State are required to report to the Official State Agency within 48 hours the source of all poultry specimens from which *S. pullorum* or *S. gallinarum* is isolated;

(v) All reports of any disease outbreak involving a disease covered under the Plan are promptly followed by an investigation by the Official State Agency to determine the origin of the infection: *Provided,* That if the origin of the infection involves another State, or if there is exposure to poultry in another

State from the infected flock, then the National Poultry Improvement Plan will conduct an investigation;

(vi) All flocks found to be infected with pullorum or typhoid are quarantined until marketed or destroyed under the supervision of the Official State Agency, or until subsequently blood tested following the procedure for reacting flocks as contained in § 145.14(a)(5), and all birds fail to demonstrate pullorum or typhoid infection;

(vii) All poultry, including exhibition, exotic, and game birds, but excluding waterfowl, going to public exhibition shall come from U.S. Pullorum-Typhoid Clean or equivalent flocks, or have had a negative pullorum- typhoid test within 90 days of going to public exhibition;

(viii) Discontinuation of any of the conditions or procedures described in paragraphs (b)(3)(i), (ii), (iii), (iv), (v), (vi), and (vii) of this section, or the occurrence of repeated outbreaks of pullorum or typhoid in poultry breeding flocks, other than turkey flocks, within or originating within the State shall be grounds for the Service to revoke its determination that such conditions and procedures have been met or complied with. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity to present its views.

(4) It is a multiplier breeding flock located in a State which has been determined by the Service to be in compliance with the provisions of paragraph (b)(3) of this section, and in which pullorum disease or fowl typhoid is not known to exist nor to have existed in hatchery supply flocks, other than turkey, waterfowl, exhibition poultry, and game bird supply flocks, within the State during the preceding 12 months.

(5) It is a primary breeding flock located in a State determined to be in compliance with the provisions of paragraph (b)(4) of this section, and in which a sample of 300 birds from flocks of more than 300, and each bird in flocks of 300 or less, has been officially tested for pullorum-typhoid with no reactors: *Provided*, That a bacteriological examination monitoring program acceptable to the Official State Agency and approved by the Service may be used in lieu of blood testing.

(c) U.S. *M. Gallisepticum* Clean

(1) A flock maintained in compliance with the provisions of § 147.26 of this chapter and in which freedom from *M. gallisepticum* has been demonstrated under the criteria specified in paragraph (c)(1)(i) or (ii) of this section.

(i) It is a flock in which all birds or a sample of at least 300 birds has been tested for *M. gallisepticum* as provided in § 145.14(b) when more than 4 months of age: *Provided,* That to retain this classification, a minimum of 150 birds shall be tested at intervals of not more than 90 days: *And provided further*, That a sample comprised of less than 150 birds may be tested at any one time, with the approval of the Official State Agency and the concurrence of the Service, provided that a minimum of 150 birds is tested within each 90-day period; or

(ii) It is a multiplier breeding flock which originated as U.S. *M. Gallisepticum* Clean chicks from primary breeding flocks and from which a sample comprised of a minimum of 150 birds per flock has been tested for *M. gallisepticum* as provided in §145.14(b) when more than 4 months of age: *Provided*, That to retain this classification, the flock shall be subjected to one of the following procedures:

(A) At intervals of not more than 90 days, a sample of 75 birds, with a minimum of 30 birds per pen, whichever is greater shall be tested; or

(B) At intervals of not more than 30 days, a sample of 25 cull chicks produced from the flock shall be subjected to laboratory procedures acceptable to the Official State Agency and approved by the Service, for the detection and recovery of *M. gallisepticum;* or

(C) At intervals of not more than 30 days, egg yolk testing shall be conducted in accordance with § 147.8.

(2) A participant handling U.S. *M. Gallisepticum* Clean products shall keep these products separate from other products in a manner satisfactory to the Official State Agency: *Provided*, That U.S. *M. Gallisepticum* Clean chicks from primary breeding flocks shall be produced in incubators and hatchers in which only eggs from flocks qualified under paragraph (c)(1)(i) of this section are set.

(3) U.S. *M. Gallisepticum* Clean chicks shall be boxed in clean boxes and delivered in trucks that have been cleaned and disinfected as described in § 147.24(a) of this chapter.

(d) U.S. Sanitation Monitored

This program is intended to be the basis from which the breeding-hatching industry may conduct a program for the prevention and control of Salmonellosis. It is intended to reduce the incidence of Salmonella organisms in hatching eggs and chicks through an effective and practical sanitation program at the breeder farm and in the hatchery. This will afford other segments of the poultry industry an opportunity to reduce the incidence of Salmonella in their products.

(1) A flock and the hatching eggs and chicks produced from it which have met the following requirements as determined by the Official State Agency:

(i) The flock shall originate from a source where sanitation and management practices, as outlined in § 145.33(d)(1) of this paragraph, are conducted;

(ii) The flock is maintained in compliance with §§ 147.21, 147.24 (a), and 147.26 of this chapter;

(iii) If pelletized feed contains animal protein, the protein products should be purchased from participants in the Animal Protein Products Industry (APPI) Salmonella Education/Reduction Program. The protein products must have a minimum moisture content of 14.5 percent and must have been heated throughout to a minimum temperature of 190 °F. or above, or to a minimum temperature of 165 °F. for at least 20 minutes, or to a minimum temperature of 184 °F. under 70 lbs. pressure during the manufacturing process;

(iv) If mash feed contains animal protein, the protein products should be purchased from participants in the Animal Protein Products Industry (APPI) Salmonella Education/Reduction Program;

(v) Feed shall be stored and transported in such a manner as to prevent possible contamination;

(vi) Chicks shall be hatched in a hatchery meeting the requirements of §§ 147.23 and 147.24(b) and sanitized or fumigated (see § 147.25 of this chapter);

(vii) An Authorized Agent shall take environmental samples, as described in § 147.12 of this chapter, from each flock at 4 months of age and every 90 days thereafter. An authorized laboratory for Salmonella shall examine the environmental samples bacteriologically;

(viii) Owners of flocks found infected with a paratyphoid Salmonella may vaccinate these flocks with an autogenous bacterin with a potentiating agent.[4]

(2) The Official State Agency may use the procedures described in § 147.14 of this chapter to monitor the effectiveness of the sanitation practices.

(3) In order for a hatchery to sell products of this classification, all products handled shall meet the requirements of the classification.

(4) This classification may be revoked by the Official State Agency if the participant fails to follow recommended corrective measures.

(e) U.S. *M. Synoviae* Clean

(1) A flock maintained in compliance with the provisions of § 147.26 and in which freedom from *M. synoviae* has been demonstrated under the criteria specified in paragraph (e)(1)(i) or (ii) of this section.

(i) It is a flock in which a minimum of 300 birds has been tested for *M. synoviae* as provided in § 145.14(b) when more than 4 months of age: *Provided*, That to retain this classification, a sample of at least 150 birds shall be tested at intervals of not more than 90 days: *And provided further*, That a sample comprised of less than 150 birds may be tested at any one time, with the approval of the Official State Agency and the concurrence of the Service, provided that a minimum of 150 birds is tested within each 90-day period; or

(ii) It is a multiplier breeding flock which originated as U.S. *M. synoviae* Clean chicks from primary breeding flocks and from which a sample comprised of a minimum of 75 birds has been tested for *M. synoviae* as provided in § 145.14(b) when more than 4 months of age: *Provided*, That to retain this classification, the flock shall be subjected to one of the following procedures:

(A) At intervals of not more than 90 days, a sample of 50 birds shall be tested: *Provided*, That a sample of less than 50 birds may be tested at any one time, provided that a minimum of 30 birds per flock with a minimum of 15 birds per pen, whichever is greater, is tested each time and a total of at least 50 birds is tested within each 90-day period; or

(B) At intervals of not more than 30 days, egg yolk testing shall be conducted in accordance with § 147.8.

(2) A participant handling U.S. *M. Synoviae* Clean products shall keep these products separate from other products in a manner satisfactory to the Official State Agency: *Provided*, That U.S. *M. Synoviae* Clean chicks from primary breeding flocks shall be produced in incubators and hatchers in which only eggs from flocks qualified under paragraph (e)(1)(i) or (ii) of this section are set.

(3) U.S. *M. synoviae* Clean chicks shall be boxed in clean boxes and delivered in trucks that have been cleaned and disinfected as described in § 147.24(a).

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[4]Preparation and use of this type of vaccine may be regulated by State statutes.

(f) U.S. *M. Gallisepticum* Clean Started Poultry

(1) A flock which originated from U.S. *M. Gallisepticum* Clean breeding flocks and was hatched in a hatchery approved by the Official State Agency for the production of U.S. *M. Gallisepticum* Clean chicks.
(2) All other poultry on the premises of the candidate flock must originate from U.S. *M. Gallisepticum* Clean sources.
(3) The flock is maintained in compliance with the provisions of § 147.26.
(4) The flock's freedom from *M. gallisepticum* is demonstrated by a negative blood test, as provided in § 145.14(b), of a sample of 75 birds, with a minimum of 50 birds per poultry house, between 15–20 days prior to the flock being moved to laying quarters.
(5) Started poultry shall be delivered to and from the farm premises in crates and vehicles which have been cleaned and disinfected as described in § 147.24(a) of this chapter.

(g) U.S. *M. Synoviae* Clean Started Poultry

(1) A flock which originated from U.S. *M. Synoviae* Clean breeding flocks and was hatched in a hatchery approved by the Official State Agency for the production of U.S. *M. Synoviae* Clean chicks.
(2) All other poultry on the premises of the candidate flock must originate from U.S. *M. Synoviae* Clean sources.
(3) The flock is maintained in compliance with the provisions of § 147.26.
(4) The flock's freedom from *M. synoviae* is demonstrated by a negative blood test, as provided in § 145.14(b), of a sample of 75 birds, with a minimum of 50 birds per poultry house, between 15–20 days prior to the flock being moved to laying quarters.
(5) Started poultry shall be delivered to and from the farm premises in crates and vehicles which have been cleaned and disinfected as described in § 147.24(a) of this chapter.

(Approved by the Office of Management and Budget under control number 0579-0007)

§ 145.34 Terminology and Classification; States.

(a) U.S. Pullorum-Typhoid Clean State

(1) A State will be declared a U.S. Pullorum-Typhoid Clean State when it has been determined by the Service that:
  (i) The State is in compliance with the provisions contained in § 145.23(b)(3)(i) through (vii), § 145.33(b)(3)(i) through (vii), § 145.43(b)(3)(i) through (vi), and § 145.53(b)(3)(i) through (vii).
  (ii) No pullorum disease or fowl typhoid is known to exist nor to have existed in hatchery supply flocks within the State during the preceding 12 months: *Provided*, That pullorum disease or fowl typhoid found within the preceding 24 months in waterfowl, exhibition poultry, and game bird breeding flocks will not prevent a State, which is otherwise eligible from qualifying.

(2) Discontinuation of any of the conditions described in paragraph (a)(1)(i) of this section, or repeated outbreaks of pullorum or typhoid occur in hatchery supply flocks described in paragraph (a)(1)(ii) of this section, or if an infection spreads from the originating premises, the Service shall have grounds to revoke its determination that the State is entitled to this classification. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity for a hearing.

(b) U.S. *M. Gallisepticum* Clean State, Meat-Type Chickens

(1) A State will be declared a U.S. *M. Gallisepticum* Clean State, Meat-Type Chickens, when it has been determined by the Service that:

(i) No *M. gallisepticum* is known to exist nor to have existed in meat-type chicken breeding flocks in production within the State during the preceding 12 months;

(ii) All meat-type chicken breeding flocks in production are classified as U.S. *M. gallisepticum* Clean or have met equivalent requirements for *M. gallisepticum* control under official supervision;

(iii) All hatcheries within the State which handle products from meat-type chicken breeding flocks only handle products which are classified as U.S. *M. Gallisepticum* Clean or have met equivalent requirements for *M. gallisepticum* control under official supervision;

(iv) All shipments of products from meat-type chicken breeding flocks other than those classified as U.S. *M. Gallisepticum* Clean, or equivalent, into the State are prohibited;

(v) All persons performing poultry disease diagnostic services within the State are required to report to the Official State Agency within 48 hours the source of all specimens from chickens from meat-type chicken breeding flocks that have been identified as being infected with *M. gallisepticum*;

(vi) All reports of *M. gallisepticum* infection in chickens from meat-type chicken breeding flocks are promptly followed by an investigation by the Official State Agency to determine the origin of the infection;

(vii) All chickens from meat-type chicken breeding flocks found to be infected with *M. gallisepticum* are quarantined until marketed under supervision of the Official State Agency.

(2) Discontinuation of any of the conditions described in paragraph (b)(1) of this section, or if repeated outbreaks of *M. gallisepticum* occur in meat-type chicken breeding flocks described in paragraph (b)(1)(ii) of this section, or if an infection spreads from the originating premises, the Service shall have grounds to revoke its determination that the State is entitled to this classification. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity for a hearing.

Subpart D—Special Provisions for Turkey Breeding Flocks and Products

§ 145.41 Definitions.

Except where the context otherwise requires, for the purposes of this subpart the following terms shall be construed, respectively, to mean:

Poults Newly hatched turkeys which have not been fed or watered.

§ 145.42 Participation.

(a) Participating turkey flocks, and the eggs and poults produced from them, shall comply with the applicable general provisions of Subpart A of this part and the special provisions of this Subpart D.
(b) The minimum weight of turkey hatching eggs shipped interstate shall be 2 ounces each for small varieties and 2 1/2 ounces each for other varieties, unless otherwise specified by the purchaser of the eggs.
(c) Hatching eggs shall be fumigated (see § 147.25 of this chapter) or otherwise sanitized.

§ 145.43 Terminology and Classification; Flocks and Products.

Participating flocks, and the eggs and poults produced from them, which have met the respective requirements specified in this section may be designated by the following terms and the corresponding designs illustrated in § 145.10:

(a) [Reserved]

(b) U.S. Pullorum-Typhoid Clean A flock in which freedom from pullorum and typhoid has been demonstrated to the Official State Agency under the criteria in one of the following paragraphs (b)(1) through (5) of this section: *Provided*, That a flock qualifying by means of a blood test shall be tested within the past 12 months, except that the retesting of a participating flock which is retained for more than 12 months shall be at the discretion of the Official State Agency with the concurrence of the Service. (See § 145.14 relating to the official blood test where applicable.)

(1) It has been officially blood tested with no reactors.
(2) It is a multiplier breeding flock, or a breeding flock composed of progeny of a primary breeding flock which is intended solely for the production of multiplier breeding flocks, and meets the following specifications as determined by the Official State Agency and the Service:
    (i) The flock is located in a State where all persons performing poultry disease diagnostic services within the State are required to report to the Official State Agency within 48 hours the source of all poultry specimens from which *S. pullorum* or *S. gallinarum* is isolated;

(ii) The flock is composed entirely of birds that originated from U.S. Pullorum-Typhoid Clean breeding flocks or from flocks that met equivalent requirements under official supervision; and

(iii) The flock is located on a premises where either no poultry or a flock not classified as U.S. Pullorum-Typhoid Clean were located the previous year; *Provided*, That an Authorized Agent must blood test up to 300 birds per flock, as described in § 145.14, if the Official State Agency determines that the flock has been exposed to pullorum-typhoid. In making determinations of exposure and setting the number of birds to be blood tested, the Official State Agency shall evaluate the results of any blood tests, described in § 145.14(a)(1) of this part, that were performed on an unclassified flock located on the premises during the previous year; the origins of the unclassified flock; and the probability of contacts between the flock for which qualification is being sought and (a) infected wild birds, (b) contaminated feed or waste, or (c) birds, equipment, supplies, or personnel from flocks infected with pullorum-typhoid.

(3) It is a multiplier breeding flock, or a breeding flock composed of progeny of a primary breeding flock which is intended solely for the production of multiplier breeding flocks, that originated from U.S. Pullorum-Typhoid Clean breeding flocks or from flocks that met equivalent requirements under official supervision, and is located in a State in which it has been determined by the Service that:

(i) All turkey hatcheries within the State are qualified as "National Plan Hatcheries" or have met equivalent requirements for pullorum-typhoid control under official supervision;

(ii) All turkey hatchery supply flocks within the State are qualified as U.S. Pullorum-Typhoid Clean or have met equivalent requirements for pullorum-typhoid control under official supervision: *Provided*, That if other domesticated fowl, except waterfowl, are maintained on the same premises as the participating flock, freedom from pullorum-typhoid infection shall be demonstrated by an official blood test of each of these fowl;

(iii) All shipments of products other than U.S. Pullorum-Typhoid Clean, or equivalent, into the State are prohibited;

(iv) All persons performing poultry disease diagnostic services within the State are required to report to the Official State Agency within 48 hours the source of all poultry specimens from which *S. pullorum* or *S. gallinarum* is isolated;

(v) All reports of any disease outbreak involving a disease covered under the Plan are promptly followed by an investigation by the Official State Agency to determine the origin of the infection: *Provided,* That if the origin of the infection involves another State, or if there is exposure to poultry in another State from the infected flock, then the National Poultry Improvement Plan will conduct an investigation;

(vi) All flocks found to be infected with pullorum or typhoid are quarantined until marketed or destroyed under the supervision of the Official State Agency, or until subsequently blood tested, following the procedure for reacting flocks as contained in § 145.14(a)(5), and all birds fail to demonstrate pullorum or typhoid infection;

(vii) [Reserved]

(viii) Discontinuation of any of the conditions or procedures described in paragraphs (b)(3)(i), (ii), (iii), (iv), (v), and (vi) of this section, or the occurrence of repeated outbreaks of pullorum or typhoid in turkey breeding flocks within or originating within the State shall be grounds for the Service to revoke its determination that such conditions and procedures have been met or complied with. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity to present its views.

(4) It is a multiplier breeding flock located in a State which has been determined by the Service to be in compliance with the provisions of paragraph (b)(3) of this section and in which pullorum disease or fowl typhoid is not known to exist nor to have existed in turkey hatchery supply flocks within the State during the preceding 24 months.

(5) It is a primary breeding flock located in a State determined to be in compliance with the provisions of paragraph (b)(4), of this section and in which a sample of 300 birds from flocks of more than 300, and each bird in flocks of 300 or less, has been officially tested for pullorum-typhoid with no reactors: *Provided*, That a bacteriological examination monitoring program acceptable to the Official State Agency and approved by the Service may be used in lieu of blood testing.

(c) U.S. *M. Gallisepticum* Clean

(1) A flock maintained in accordance with the conditions and procedures described in § 147.26 of this chapter, and in which no reactors are found when a random sample of at least 10 percent of the birds in the flock, or 300 birds in flocks of more than 300 and each bird in flocks of 300 or less, is tested when more than 12 weeks of age, in accordance with the procedures described in § 145.14(b): *Provided*, That to retain this classification, a minimum of 30 samples from male flocks and 60 samples from female flocks shall be retested at 28–30 weeks of age.

(2) A flock qualified as U.S. *M. Gallisepticum* Clean may retain the classification through its first egg-laying cycle, provided it is maintained in isolation and no evidence of *M. gallisepticum* infection is revealed. A flock which is molted following completion of an egg-laying cycle and subsequently brought back into production, shall be retested within 2 weeks prior to production, as described in paragraph (c)(1) of this section. A State inspector shall visit with the owner or manager of each flock at least once during each laying cycle to discuss and ascertain whether the applicable conditions outlined in § 147.26 of this chapter are being met. If a flock proves to be infected with *M. gallisepticum*, it shall lose this classification.

(3) In order to sell hatching eggs or poults of this classification, all hatching eggs and poults handled by the participant must be of this classification.

(d) U.S. *M. Meleagridis* Clean

(1) A flock in which freedom from *M. meleagridis* has been demonstrated under the following criteria:
  (i) A sample of 60 birds from each flock has been tested for *M. meleagridis* when more than 12 weeks of age: *Provided*, That to retain this classification, a minimum of 30 samples from male flocks and 60 samples from female flocks shall be retested at 28–30 weeks of age and at 4–6 week intervals thereafter.

(2) The official blood tests for *M. meleagridis* shall be the serum plate agglutination test, the tube agglutination test, or the microagglutination test. The hemagglutination inhibition (HI) test, microhemagglutination inhibition test, serum plate dilution test, microagglutination test and the enzyme-labeled immunosorbent assay (ELISA)[5] test may be used as supplemental tests to determine the status of the flock, in accordance with § 147.6(b).

(3) The tests shall be conducted using *M. meleagridis* antigens and the protocols for testing approved by the Department or the Official State Agency.

(4) When reactors to the official test are found and can be identified, 10 tracheal swabs and/or vaginal or phallus swabs and their corresponding blood samples shall be submitted to a laboratory for serological and cultural examination. If reactors cannot be identified, at least 30 tracheal swabs and/or vaginal or phallus swabs and their corresponding blood samples shall be submitted. In a flock with a low reactor rate (less than 5 reactors), the reactors may be submitted to the laboratory within 10 days for serology, necropsy, and thorough bacteriological examination.

(5) If a mycoplasma is isolated, the organism must be serotyped. If *M. meleagridis* is isolated, the flock shall be considered infected.

(e) U.S. *M. synoviae* Clean

(1) All birds, or a sample of at least 100 birds from flocks of more than 100 and each bird in flocks of 100 or less, have been tested for *M. synoviae* when more than 12 weeks of age in accordance with the procedures in § 145.14(b): *Provided*, That to retain this classification a minimum of 30 samples from male flocks and 60 samples from female flocks shall be retested at 28–30 weeks of age and at 4–6 week intervals thereafter.

(2) When reactors to the official test are found and can be identified, tracheal swabs and their corresponding blood samples from 10 (all if fewer than 10) reacting birds shall be submitted to an authorized laboratory for serological and cultural examination. If reactors cannot be identified, at least 30 tracheal swabs and their corresponding blood samples shall be submitted. In a flock with a low reactor rate (less than five reactors) the reactors may be submitted to the laboratory within 10 days for serology, necropsy, and thorough bacteriological examination. When

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[5]See footnote 3 to § 145.14(b)(1) of this part.
A.A. Ansari, R.F. Taylor, T.S. Chang, "Application of Enzyme-Linked Immunosorbent Assay for Detecting Antibody to Mycoplasma gallisepticum Infections in Poultry," Avian Diseases, Vol. 27, No. 1, pp. 21–35, January–March 1983; and
H.M. Opitz, J.B. Duplessis, and M.J. Cyr, "Indirect Micro-Enzyme-Linked Immunosorbent Assay for the Detection of Antibodies to Mycoplasma synoviae and M. gallisepticum," Avian Diseases, Vol. 27, No. 3, pp. 773–786, July–September 1983; and
H.B. Ortmayer and R. Yamamoto, "Mycoplasma meleagridis Antibody Detection by Enzyme-Linked Immunosorbent Assay (ELISA)," Proceedings, 30th Western Poultry Disease Conference, pp. 63–66, March 1981.

reactors to the official test are found, the procedures outlined in § 147.6 will be used to determine the status of the flock.

(3) Flocks located on premises which, during 3 consecutive years, have contained breeding flocks qualified as U.S. M. Synoviae Clean, as described in paragraph (e)(1) above, may qualify for this classification by a negative blood test of at least 100 birds from flocks of more than 100 and each bird in flocks of 100 or less, when more than 12 weeks of age, and by testing a minimum of 30 samples from male flocks and 60 samples from female flocks at 28–30 weeks of age and at 45 weeks of age.

(f) U.S. Sanitation Monitored, Turkeys

A flock or hatchery whose owner is controlling or reducing the level of salmonella through compliance with sanitation and management practices as described in Subpart C of Part 147 of this chapter, and where the following monitoring, testing, and management practices are conducted:

(1) Hatchery debris (dead germ hatching eggs, fluff, and meconium collected by sexors), a sample of the poults that died within 10 days after hatching, or both, from each candidate breeding flock produced by a primary breeder, are examined bacteriologically at an authorized laboratory for Salmonella.

(2) The poults for the candidate breeding flock are placed in a building that has been cleaned, disinfected, and examined bacteriologically for the presence of Salmonella by an Authorized Agent, as described in § 147.12 of this chapter.

(3) Feed for turkeys in the candidate breeding flock shall meet the following requirements:

(i) All feed manufactured in pellet form must contain a minimum moisture content of 14.5 percent and must have been heated throughout to a minimum temperature of 190 °F. or above, or to a minimum temperature of 165 °F. for at least 20 minutes, or to a minimum temperature of 184 °F. under 70 lbs. pressure during the manufacturing process.

(ii) Initial feed (for newborn poults to 2 weeks of age) shall be manufactured in pellet form, either with no animal protein or with animal protein products produced under the Animal Protein Products Industry Salmonella Education/ Reduction Program.

(iii) Succeeding feed (for turkeys 2 weeks or older) shall be as described in (f)(3)(ii) of this section, mash that contains no animal protein products, or mash that contains an animal protein products supplement that has been manufactured in pellet form and crumbled.

(4) Environmental samples shall be taken by an Authorized Agent, as described in § 147.12 of this chapter, from each flock at 12–20 weeks of age and examined bacteriologically at an authorized laboratory for Salmonella.

(5) Owners of flocks found infected with a paratyphoid Salmonella may vaccinate these flocks with an autogenous bacterin with a potentiating agent.[6]

(6) Environmental samples shall be taken by an Authorized Agent, as described in § 147.12 of this chapter, from each flock at 35–50 weeks of age and from each molted flock at midlay, and examined bacteriologically at an authorized laboratory for Salmonella.

[6]Preparation and use of this type of vaccine may be regulated by state statutes.

(7) Environmental samples shall be taken, by an Authorized Agent using the procedures described in § 147.12 of this chapter, from the laying house after the flock is removed, and examined bacteriologically at an authorized laboratory for Salmonella.

(8) Hatchery debris (dead germ hatching eggs, fluff, and meconium collected by sexors), a sample of the poults that died within 10 days after hatching, or both shall be cultured from poults produced from hatching eggs from each flock, as a means of evaluating the effectiveness of the control procedures.

§ 145.44 Terminology and Classification; States

(a) U.S. Pullorum-Typhoid Clean State

(1) A State will be declared a U.S. Pullorum-Typhoid Clean State when it has been determined by the Service that:

(i) The State is in compliance with the provisions contained in § 145.23(b)(3)(i) through (vii), § 145.33(b)(3)(i) through (vii), § 145.43(b)(3)(i) through (vi), and § 145.53(b)(3)(i) through (vii).

(ii) No pullorum disease or fowl typhoid is known to exist nor to have existed in hatchery supply flocks within the State during the preceding 12 months: *Provided*, That pullorum disease or fowl typhoid found within the preceding 24 months in waterfowl, exhibition poultry, and game bird breeding flocks will not prevent a State, which is otherwise eligible, from qualifying.

(2) Discontinuation of any of the conditions described in paragraph (a)(1)(i) of this section, or repeated outbreaks of pullorum or typhoid occur in hatchery supply flocks described in paragraph (a)(1)(ii) of this section, or if an infection spreads from the originating premises, the Service shall have grounds to revoke its determination that the State is entitled to this classification. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity for a hearing.

(b) U.S. Pullorum-Typhoid Clean State, Turkeys

(1) A State will be declared a U.S. Pullorum-Typhoid Clean State, Turkeys, when it has been determined by the Service that:

(i) The State is in compliance with the provisions contained in § 145.43(b)(3)(i) through (vi).

(ii) No pullorum disease or fowl typhoid is known to exist nor to have existed in turkey hatchery supply flocks within the State during the preceding 24 months.

(2) Discontinuation of any of the conditions described in paragraph (b)(1)(i) of this section, or repeated outbreaks of pullorum or typhoid occur in hatchery supply flocks described in paragraph (b)(1)(ii) of this section, or if an infection spreads from the originating premises, Service shall have grounds to revoke its determination that the State is entitled to this classification. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity for a hearing.

(c) U.S. *M. Gallisepticum* Clean State, Turkeys

(1) A State will be declared a U.S. *M. Gallisepticum* Clean State, Turkeys when it has been determined by the Service that:

(i) No *M. gallisepticum* is known to exist nor to have existed in turkey breeding flocks in production within the State during the preceding 12 months;

(ii) All turkey breeding flocks in production are classified as U.S. *M. Gallisepticum* Clean or have met equivalent requirements for *M. gallisepticum* control under official supervision;

(A) At intervals of not more than 90 days, a random sample of serum or egg yolk from at least 2 percent of the birds in the flock, with a minimum of 30 birds per pen, shall be tested; or

(iii) All turkey hatcheries within the State handle products which are classified as U.S. *M. Gallisepticum* Clean or have met equivalent requirements for *M. gallisepticum* control under official supervision;

(iv) All shipments of turkey products other than those classified as U.S. *M. Gallisepticum* Clean, or equivalent, into the State are prohibited;

(v) All persons performing poultry disease diagnostic services within the State are required to report to the Official State Agency within 48 hours the source of all turkey specimens that have been identified as being infected with *M. gallisepticum*;

(vi) All reports of *M. gallisepticum* infection in turkeys are promptly followed by an investigation by the Official State Agency to determine the origin of the infection;

(vii) All turkey flocks found to be infected with *M. gallisepticum* are quarantined until marketed under supervision of the Official State Agency.

(2) Discontinuation of any of the conditions described in paragraph (c)(1) of this section, or if repeated outbreaks of *M. gallisepticum* occur in turkey breeding flocks described in paragraph (c)(1)(ii) of this section, or if an infection spreads from the originating premises, the Service shall have grounds to revoke its determination that the State is entitled to this classification. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity for a hearing.

(3) If a State retains this status for 2 or more years, individual breeding flocks in the State may qualify for an *M. gallisepticum* classification based on a negative test of a sample of 100 birds.

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Subpart E—Special Provisions for Waterfowl, Exhibition Poultry, and Game Bird Breeding Flocks and Products

§ 145.51 Definitions.

Except where the context otherwise requires, for the purposes of this subpart the following terms shall be construed, respectively, to mean:

Exhibition Poultry — Domesticated fowl which are bred for the combined purposes of meat or egg production and competitive showing.

Game birds — Domesticated fowl such as pheasants, partridge, quail, grouse, and guineas, but not doves and pigeons.

Waterfowl — Domesticated fowl that normally swim, such as ducks and geese.

§ 145.52 Participation.

Participating flocks of waterfowl, exhibition poultry, and game birds, and the eggs and baby poultry produced from them shall comply with the applicable general provisions of Subpart A of this part and the special provisions of this Subpart E.

(a) Started poultry shall lose their identity under Plan terminology when not maintained by Plan participants under the conditions prescribed in § 145.5(a).

(b) Hatching eggs produced by primary breeding flocks shall be fumigated (see § 147.25 of this chapter) or otherwise sanitized.

§ 145.53 Terminology and Classification; Flocks and Products.

Participating flocks, and the eggs and baby poultry produced from them, which have met the respective requirements specified in this section may be designated by the following terms and the corresponding designs illustrated in § 145.10.

(a) U.S. Approved — All birds in the breeding flock observed by Authorized Agents or State Inspectors are found to conform with the criteria for the breed represented, as contained in the Standard of Perfection[7] or the breeder's specifications for the stock represented in the flock, and such specifications are on file with the Official State Agency.

(b) U.S. Pullorum-Typhoid Clean — A flock in which freedom from pullorum and typhoid has been demonstrated to the Official State Agency under the criteria in one of the following paragraphs (b)(1) through (5) of this section (See § 145.14 relating to the official blood test where applicable.):

(1) It has been officially blood tested within the past 12 months with no reactors.

[7]Published by the American Poultry Association, Inc.

(2) It is a multiplier breeding flock, or a breeding flock composed of progeny of a primary breeding flock which is intended solely for the production of multiplier breeding flocks, and meets the following specifications as determined by the Official State Agency and the Service:

(i) The flock is located in a State where all persons performing poultry disease diagnostic services within the State are required to report to the Official State Agency within 48 hours the source of all poultry specimens from which *S. pullorum* or *S. gallinarum* is isolated;

(ii) The flock is composed entirely of birds that originated from U.S. Pullorum-Typhoid Clean breeding flocks or from flocks that met equivalent requirements under official supervision; and

(iii) The flock is located on a premises where either no poultry or a flock not classified as U.S. Pullorum-Typhoid Clean were located the previous year: *Provided*, That an Authorized Agent must blood test up to 300 birds per flock, as described in § 145.14, if the Official State Agency determines that the flock has been exposed to pullorum-typhoid. In making determinations of exposure and setting the number of birds to be blood tested, the Official State Agency shall evaluate the results of any blood tests, described in § 145.14(a)(1) of this part, that were performed on an unclassified flock located on the premises during the previous year; the origins of the unclassified flock; and the probability of contacts between the flock for which qualification is being sought and (a) infected wild birds, (b) contaminated feed or waste, or (c) birds, equipment, supplies, or personnel from flocks infected with pullorum-typhoid.

(3) It is a multiplier breeding flock that originated from U.S. Pullorum-Typhoid Clean breeding flocks or from flocks that met equivalent requirements under official supervision, and is located in a State in which it has been determined by the Service that:

(i) All hatcheries, except turkey hatcheries, within the State are qualified as "National Plan Hatcheries" or have met equivalent requirements for pullorum-typhoid control under official supervision;

(ii) All hatchery supply flocks, except turkey flocks, within the State, are qualified as U.S. Pullorum-Typhoid Clean or have met equivalent requirements for pullorum-typhoid control under official supervision: *Provided*, That if other domesticated fowl, except waterfowl, are maintained on the same premises as the participating flock, freedom from pullorum-typhoid infection shall be demonstrated by an official blood test of each of these fowl;

(iii) All shipments of products other than U.S. Pullorum-Typhoid Clean, or equivalent, into the State are prohibited;

(iv) All persons performing poultry disease diagnostic services within the State are required to report to the Official State Agency within 48 hours the source of all poultry specimens from which *S. pullorum* or *S. gallinarum* is isolated;

(v) All reports of any disease outbreak involving a disease covered under the Plan are promptly followed by an investigation by the Official State Agency to determine the origin of the infection: *Provided,* That if the origin of the infection involves another State, or if there is exposure to poultry in another State from the infected flock, then the National Poultry Improvement Plan will conduct an investigation;

(vi) All flocks found to be infected with pullorum or typhoid are quarantined until marketed or destroyed under the supervision of the Official State Agency, or until subsequently blood tested, following the procedure for reacting flocks as contained in § 145.14(a)(5), and all birds fail to demonstrate pullorum or typhoid infection;

(vii) All poultry, including exhibition, exotic, and game birds, but excluding waterfowl, going to public exhibition shall come from U.S. Pullorum-Typhoid Clean or equivalent flocks, or have had a negative pullorum-typhoid test within 90 days of going to public exhibition;

(viii) Discontinuation of any of the conditions or procedures described in paragraphs (b)(3)(i), (ii), (iii), (iv), (v), (vi), and (vii) of this section, or the occurrence of repeated outbreaks of pullorum or typhoid in poultry breeding flocks, other than turkey flocks, within or originating within the State shall be grounds for the Service to revoke its determination that such conditions and procedures have been met or complied with. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity to present its views.

(4) It is a multiplier breeding flock located in a State which has been determined by the Service to be in compliance with the provisions of paragraph (b)(3) of this section, and in which pullorum disease or fowl typhoid is not known to exist nor to have existed in hatchery supply flocks, other than turkey flocks, within the State during the preceding 24 months.

(5) It is a primary breeding flock located in a State determined to be in compliance with the provisions of paragraph (b)(4) of this section, and in which a sample of 300 birds from flocks of more than 300, and each bird in flocks of 300 or less, has been officially tested for pullorum-typhoid within the past 12 months with no reactors: *Provided*, That a bacteriological examination monitoring program or serological examination monitoring program for game birds acceptable to the Official State Agency and approved by the Service may be used in lieu of annual blood testing: *And provided further*, That when a flock is a waterfowl or exhibition poultry primary breeding flock located in a State which has been deemed to be a U.S. Pullorum-Typhoid Clean State for the past three years, and during which time no isolation of pullorum or typhoid has been made that can be traced to a source in that State, a bacteriological examination monitoring program or a serological examination monitoring program acceptable to the Official State Agency and approved by the Service may be used in lieu of annual blood testing.

(c) U.S. *M. Gallisepticum* Clean

(1) A flock maintained in compliance with the provisions of § 147.26 of this chapter and in which freedom from *M. gallisepticum* has been demonstrated under the criteria specified in paragraph (c)(1)(i) or (ii) of this section.

(i) It is a flock in which all birds or a sample of at least 300 birds has been tested for *M. gallisepticum* as provided in § 145.14(b) when more than 4 months of age or upon reaching sexual maturity: *Provided*, That to retain this classification, a random sample of serum or egg yolk from at least 5 percent of the birds in the flock, but at least 30 birds, shall be tested at intervals of not more than 90 days: *And provided further*, That a sample comprised of less than 5 percent may be tested at any one time, with the approval of the Official State Agency and the concurrence of the Service, provided that a total of at least 5 percent of the birds in the flock, but at least 30 birds, is tested within each 90-day period; or

(ii) It is a multiplier breeding flock which originated as U.S. *M. Gallisepticum* Clean baby poultry from primary breeding flocks and a random sample comprised of 50 percent of the birds in the flock, with a maximum of 200 birds and a minimum of 30 birds per flock, has been tested for *M. gallisepticum* as provided in § 145.14(b) when more than 4 months of age or upon reaching sexual maturity: *Provided*, That to retain this classification, the flock shall be subjected to one of the following procedures:

(A) At intervals of not more than 90 days, a random sample of serum or egg yolk from at least 2 percent of the birds in the flock, with a minimum of 30 birds per pen, shall be tested; or

(B) At intervals of not more than 30 days, a sample of 25 cull baby poultry produced from the flock shall be subjected to laboratory procedures acceptable to the Official State Agency and approved by the Service, for the detection and recovery of *M. gallisepticum*.

(2) A participant handling U.S. *M. Gallisepticum* Clean products shall keep these products separate from other products in a manner satisfactory to the Official State Agency: *Provided*, That U.S. *M. Gallisepticum* Clean baby poultry from primary breeding flocks shall be produced in incubators and hatchers in which only eggs from flocks qualified under paragraph (c)(1)(i) of this section are set.

(3) U.S. *M. Gallisepticum* Clean baby poultry shall be boxed in clean boxes and delivered in trucks that have been cleaned and disinfected as described in § 147.24(a) of this chapter.

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§ 145.54 Terminology and Classification; States.

(a) U.S. Pullorum-Typhoid Clean State

(1) A State will be declared a U.S. Pullorum-Typhoid Clean State when it has been determined by the Service that:

(i) The State is in compliance with the provisions contained in § 145.23(b)(3)(i) through (vii), § 145.33(b)(3)(i) through (vii), § 145.43(b)(3)(i) through (vi), and § 145.53(b)(3)(i) through (vii);

(ii) No pullorum disease or fowl typhoid is known to exist nor to have existed in hatchery supply flocks within the State during the preceding 12 months: *Provided*, That pullorum disease or fowl typhoid found within the preceding 24 months in waterfowl, exhibition poultry, and game bird breeding flocks will not prevent a State, which is otherwise eligible, from qualifying.

(2) Discontinuation of any of the conditions described in paragraph (a)(1)(i) of this section, or repeated outbreaks of pullorum or typhoid occur in hatchery supply flocks described in paragraph (a)(1)(ii) of this section, or if an infection spreads from the originating premises, the Service shall have grounds to revoke its determination that the State is entitled to this classification. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity for a hearing.

PART 146—[Reserved]

PART 147—Auxiliary Provisions On National Poultry Improvement Plan

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Authority: 7 U.S.C. 429; 7 CFR 2.17, 2.51, and 371.2(d).

§ 147.1 The Standard Tube Agglutination Test.[1]

(a) The blood samples should be collected and delivered as follows:

(1) The blood samples should be taken by properly qualified and authorized persons only, and in containers provided by the laboratory. The containers should be stout-walled test tubes, preferably 3/8 by 3 inches, without lip, or small well-selected medicine vials, which have been thoroughly cleaned and dried in a hot-air drying oven. If stoppers are used, they should be thoroughly cleaned and dried.

(2) Sufficient blood should be procured by making a small incision in the large median wing vein with a small sharp lancet and allowing the blood to run into the tube, or by the use of a small syringe (with 20 or 21 gauge needle) which is properly cleansed between bleedings with physiological saline solution. To facilitate the separation of the serum, the tubes should be placed in a slanted position until the blood has solidified. After the blood has completely clotted, they should be packed and shipped by mail (special delivery), rapid express, or by messenger, to the laboratory. All labeling must be clear and permanent, and may be done with a suitable pencil on etched portions of the tube, or by means of fast-gum labels.

(3) The blood samples must reach the laboratory in a fresh and unhemolyzed condition. Hemolyzed samples should be rejected. It is imperative, therefore, to cool the tubes immediately after slanting and clotting, and unless they reach the laboratory within a few hours, to pack them with ice in special containers, or use some other cooling system which will insure their preservation during transportation. In severe cold seasons, extreme precautions must be exercised to prevent freezing and consequent laking. The samples must be placed in cold (5 to 10 °C.) storage, immediately upon arrival at the laboratory.

(b) The antigen shall consist of representative strains of *S. pullorum* which are of known antigenic composition, high agglutinability, but are not sensitive to negative and nonspecific sera. The stock cultures may be maintained satisfactorily by transferring to new sloped agar at least once a month and keeping at 18 to 25 °C. (average room temperature) in a dark closet or chest, following incubation for from 24 to 36 hours at 37 °C. The antigenic composition and purity of the stock cultures should be checked consistently.

(c) A medium which has been used satisfactorily has the following composition:

Water	1,000 cc.
Difco beef extract	4 g. (0.4 percent)
Difco Bacto-peptone	10 g. (1.0 percent)
Difco dry-granular agar	20 g. (2.0 percent)
Reaction—Ph 6.8 to 7.2	

[1]The procedure described is a modification of the method reported in the Proceedings of the U.S. Live Stock Sanitary Association, November 30 to December 2, 1932, pp. 487 to 491.

(d) Large 1-inch test tubes, Kolle flasks, or Blake bottles should be streaked liberally over the entire agar surface with inoculum from 48-hour slant agar cultures prepared from the stock cultures of the selected strains. The antigen-growing tubes or bottles should be incubated 48 hours at 37 °C., and the surface growth washed off with sufficient phenolized (0.5 percent) saline (0.85 percent) solution to make a heavy suspension. The suspension should be filtered free of clumps through a thin layer of absorbent cotton in a Buchner funnel with the aid of suction. The antigens of the separate strains should be combined in equal volume-density and stored in the refrigerator (5 to 10 °C.) in tightly stoppered bottles.

(e) Thiosulfate-Glycerin (TG) medium may be used as an alternate medium for the preparation of tube agglutination antigen. The TG medium, formerly used for the preparation of stained, whole-blood antigen, is described in more detail in the article by A. D. MacDonald, *Recent Developments in Pullorum Antigen for the Rapid, Whole-Blood Test*, Report of the Conference of the National Poultry Improvement Plan, pages 122–127, 1941. This medium provides a tube antigen of excellent specificity and greatly increases the yield of antigen from a given amount of medium. The TG medium has the following composition:

Beef infusion	1,000 cc.
Difco Bacto-peptone	20 g. (2.0 percent)
Sodium thiosulfate	5 g. (0.5 percent)
Ammonium chloride	5 g. (0.5 percent)
Glycerin, U.S.P.(95 percent)	20 cc.(2.0 percent)
Difco dry-granular agar	30 g. (3.0 percent)
Reaction—Ph 6.8 to 7.2	

Large 1-inch test tubes, Kolle flasks, Blake bottles, or Erlenmeyer flasks should be seeded over the entire agar surface with inoculum from 24-hour beef infusion broth cultures prepared from the stock cultures of the selected strains. The antigen-growing tubes or bottles should be incubated 96 hours at 37 °C., and the surface growth washed off with sufficient phenolized (0.5 percent) saline (0.85 percent) solution to make a heavy suspension. The suspension should be filtered free of clumps through a thin layer of absorbent cotton in a Buchner funnel with the aid of suction. The antigen then should be centrifuged. The mass of bacteria should be removed from the centrifuge tubes or bowl and resuspended in saline (0.85 percent) solution containing 0.5 percent phenol. After the bacterial mass has been uniformly suspended in the diluent, it should be again passed through a cotton pad in a Buchner funnel without the aid of suction. The antigens of the separate strains should be combined in equal volume-density and stored in the refrigerator (5 to 10 °C.) in tightly stoppered bottles.

(f) The diluted antigen to be used in the routine testing should be prepared from the stock antigen by dilution of the latter with physiological (0.85 percent) saline solution containing 0.25 percent of phenol to a turbidity corresponding to 0.75–1.00 on the McFarland nephelometer scale. The hydrogen-ion concentration of the diluted antigen should be corrected to Ph 8.2 to 8.5 by the addition of dilute sodium hydroxide. New diluted antigen should be prepared each day and kept

cold. The diluted antigen may be employed in 2 cc. quantities in 4 by 1/2-inch test tubes, or 1 cc. quantities in smaller tubes, in which the final serum-antigen mixtures are made and incubated. The distribution of the antigen in the tubes may be accomplished by the use of long burettes, or special filling devices made for the purpose.

(g) The maximum serum dilution employed must not exceed 1:50 for chickens, nor 1:25 for turkeys. The available data indicate that 1:25 dilution is the most efficient. In all official reports on the blood test, the serum dilutions shall be indicated. The sera should be introduced into the agglutination tubes in the desired amounts with well-cleaned serological pipettes or special serum-delivery devices which do not permit the mixing of different sera. The antigen and serum should be well mixed before incubation. The serum and antigen mixture must be incubated for at least 20 hours at 37 °C.

(h) The results shall be recorded as:

N, or –(negative) when the serum-antigen mixture remains uniformly turbid.
P, or + (positive) when there is a distinct clumping of the antigen, and the liquid between the agglutinated particles is clear.
S, or ? (suspicious) when the agglutination is only partial or incomplete.
M, or missing, when samples listed on the original record sheet are missing.
H, or hemolyzed, when blood samples are hemolyzed and cannot be tested.
B, or broken, when sample tubes are broken and no serum can be obtained.

(Some allowance must always be made for the difference in sensitivity of different antigens and different set-ups, and therefore, a certain amount of independent, intelligent judgment must be exercised at all times. Also, the histories of the flocks require consideration. In flocks where individuals show a suspicious agglutination, it is desirable to examine representative birds bacteriologically to determine the presence or absence of *S. pullorum*.)

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§ 147.2 The Rapid Serum Test.[2]

(a) The procedure for the collection and delivery of blood samples in the rapid serum test is the same as that described in § 147.1(a).

(b) The selection and maintenance of suitable strains of *S. pullorum* and the composition of a satisfactory medium are described in § 147.1(b) and (c).

(c) Large 1-inch test tubes, Kolle flasks, or Blake bottles are streaked liberally from 48-hour slant-agar cultures prepared from stock cultures of the selected strains.

[2]The procedure described is a modification of the method reported by Runnels, Coon, Farley, and Thorpe, Amer. Vet. Med. Assoc. Jour. 70 (N.S. 23): 660–662 (1927).

(d) The antigen-growing tubes or bottles should be incubated 48 hours at 37 °C., and the surface growth washed off with a very slight amount of 12 percent solution of sodium chloride containing 0.25 to 0.5 percent phenol, filtered through lightly packed sterile absorbent cotton placed in the apex of a sterile funnel.

(e) The washings should be adjusted (using 12 percent sodium chloride containing 0.25 to 0.5 percent phenol) so that the turbidity is 50 times greater than tube 0.75 of McFarland's nephelometer, or to a reading of 7 mm. by the Gates nephelometer.

(f) The individual strain antigens should be tested with negative sera for their insensitivity and with positive sera for high agglutinability in comparison with known satisfactory antigen. The antigens of the separate strains should be combined in equal volume-density and stored in the refrigerator (5 to 10 °C.) in tightly stoppered bottles.

(g) The tests should be conducted on a suitable, smooth plate. The serum-antigen dilution should be made so that the dilution will not exceed 1:50 when compared to the standard tube agglutination test. When testing turkey blood samples, it is desirable to use a serum-antigen dilution equivalent to the 1:25 in the tube method. The serum should be added to the antigen and mixed thoroughly by use of the tip of the serum pipette. Most strong positive reactions will be plainly evident within 15 to 20 seconds. The final reading should be made at the end of 2 or 3 minutes. Heating the plate at approximately 37 °C. will hasten agglutination. Before reading, the plate should be rotated several times.

(h) The results shall be recorded as described in § 147.1(h).

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§ 147.3 The Stained-Antigen, Rapid, Whole-Blood Test.[3]

(a) The description of the preparation of antigen is not herein included because the antigen is a proprietary product produced only under license from the Secretary of Agriculture.

(b) A loop for measuring the correct quantity of blood can usually be obtained from the manufacturer of the antigen. A satisfactory loop may be made from a piece of No. 20 gauge nichrome wire, 2 1/2 inches long, at the end of which is fashioned a loop three-sixteenths of an inch in diameter. Such a loop, when filled with blood so that the blood appears to bulge, delivers 0.02 cc. A medicine dropper whose tip is adjusted to deliver 0.05 cc. is used to measure the antigen. A glass plate about 15 inches square, providing space for 48 tests, has proved satisfactory for this work. The use of such a plate enables the tester to have a number of successive test mixtures under observation without holding up the work to wait for results before proceeding to the next bird.

[3] The procedure described is a modification of the method reported by Schaffer, MacDonald, Hall, and Bunyea, Jour. Amer. Vet. Med. Assoc. 79 (N. S. 32): 236–240 (1931).

(c) A drop of antigen should be placed on the testing plate. A loop full of blood should be taken up from the wing vein. When submerged in the blood and then carefully withdrawn, the loop becomes properly filled. On looking down edgewise at the filled loop, one observes that the blood appears to bulge. The loopful of blood then should be stirred into the drop of antigen, and the mixture spread to a diameter of about 1 inch. The loop then should be rinsed in clean water and dried by touching it to a piece of clean blotting paper, if necessary. The test plate should be rocked from side to side a few times to mix the antigen and blood thoroughly, and to facilitate agglutination. The antigen should be used according to the directions of the producer.

(d) Various degrees of reaction are observed in this as in other agglutination tests. The greater the agglutinating ability of the blood, the more rapid the clumping and the larger the clumps. A positive reaction consists of a definite clumping of the antigen surrounded by clear spaces. Such reaction is easily distinguished against a white background. A somewhat weaker reaction consists of small but still clearly visible clumps of antigen surrounded by spaces only partially clear. Between this point and a negative or homogeneous smear, there sometimes occurs a very fine granulation barely visible to the naked eye; this should be disregarded in making a diagnosis. The very fine marginal clumping which may occur just before drying up is also regarded as negative. In a nonreactor, the smear remains homogeneous.

(Allowance should be made for differences in the sensitivity of different antigens and different set-ups, and therefore, a certain amount of independent, intelligent judgment must be exercised at all times. Also, the histories of the flocks require consideration. In flocks where individuals show a suspicious agglutination, it is desirable to examine representative birds bacteriologically to determine the presence or absence of *S. pullorum*.)

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§ 147.4 The Tube Agglutination Test for *S. Typhimurium.*

(a) The procedure for the collection and delivery of blood samples in the tube agglutination test for *S. typhimurium* is the same as that described in § 147.1(a).

(b) The "O" antigen should be prepared as follows:

(1) The antigen shall consist of a representative nonmotile strain of *S. typhimurium* which is of known antigenic composition and high agglutinability but is not sensitive to negative and nonspecific sera. Strain P 10 meets these requirements.

(2) The stock culture is maintained on 1 percent nutrient agar deeps, which have been incubated for 18–24 hours at 37 °C. They are stored at room temperature.

(3) A satisfactory medium used for growing the organism is veal infusion agar (Difco). It is dispensed in 50 ml. amounts into 500 ml. medicine bottles, with screw caps, and sterilized at 15 pounds pressure for 20 minutes. The bottles are then laid flat upon an even surface until the medium has solidified.

(4) The inoculum used for preparation of "O" antigen is a nonmotile strain of *S. typhimurium*. The organism is grown in veal infusion broth (Difco) for 18–24 hours at 37 °C.; then plated, for single colony isolation, on veal infusion agar plates. These plates are incubated for 18–24 hours at 37 °C. After incubation, single colonies are picked and transferred to veal infusion agar slants, which are incubated for 18–24 hours at 37 °C. After this, the cultures are tested for smoothness by using a 1:500 dilution of acriflavine.

(5) Smooth cultures are inoculated into flasks containing veal or beef infusion broth which is incubated for 18–24 hours at 37 °C. The incubated broth suspension of organisms is dispensed into the antigen bottles containing veal infusion agar. The suspension is distributed evenly over the agar surface by gently tilting the bottles from side to side. The inoculated bottles are then laid flat, agar side down, for 10–20 minutes. They are subsequently incubated, agar side upward, for 24–48 hours at 37 °C. before harvesting.

(6) The harvesting of the organism consists of washing the growth from each antigen bottle with 0.5 percent phenolized physiological saline. The bacterial suspension from each bottle is filtered through sterile milk pad filters into a large sterile container or through a thin layer of absorbent cotton in a Buchner funnel with the aid of suction. To each 100 ml. of the bacterial suspension is added additional phenol to make the final concentration 0.5 percent. The concentrated antigen is tested for sterility at intervals after 24 hours. After sterility is proved, the stock antigen is standardized to determine the density according to the McFarland nephelometer scale.

(7) The diluted antigen to be used in routine testing is prepared from stock antigen, by diluting with 0.25 percent phenolized saline, and is standardized to a turbidity corresponding to 0.75–1.00 of the McFarland nephelometer scale.

(c) The maximum serum dilution employed for the "O" antigen tube test must not exceed 1:25. In all official reports on the blood test, the serum dilutions should be indicated. The sera should be introduced into the agglutination tubes in the desired amounts with well-cleaned serological pipettes or special serum delivery devices which do not permit the mixing of different sera. The antigen and serum should be well mixed before incubation. The serum and antigen mixture must be incubated for at least 20 hours at 37 °C.

(d) The results shall be recorded as described in § 147.1(h).

§ 147.5 The Microagglutination Test for Pullorum-Typhoid.

Routinely, the microagglutination test is applied as a single-dilution test and only a single 18–24 hour reading is made.

(a) The procedure for the collection and delivery of blood samples in the microagglutination test is the same as that described in § 147.1(a). A method that has proven advantageous is to transfer the serum samples from the blood clot to a microplate as described in "Applied Microbiology," volume 24, No. 4, October 1972, pages 671–672. The dilutions are then performed according to paragraphs (d) or (e) of this section.

(b) Stained microtest antigen for pullorum-typhoid is supplied as concentrated stock suspension and must be approved by the Department.[4] Directions for diluting will be provided with the antigen. The stock as well as the diluted antigen prepared each day should be kept sealed in the dark at 5 to 10 °C. when not in use.

(c) Available data indicate that a 1:20 dilution for the microagglutination test is most efficient for the detection of pullorum-typhoid agglutinins in both chickens and turkeys. In all official reports on the blood test, the serum dilutions shall be indicated.

(d) The recommended procedure for the 1:20 dilution in the microagglutination test is as follows:

(1) Add 100 microliters (0.10 cc.) of 0.85 percent physiological saline to each well of the microplate.

(2) Using a microdiluter or a multimicrodiluter handle fitted with twelve 10 microliter microdiluters, transfer 10 microliters (0.01 cc.) of the serum sample from the collected specimen to the corresponding well of the microplate. This is accomplished by touching the surface of the serum sample with the microdiluter and then transferring and mixing with the diluent in the microplate well. The microdiluter is removed, blotted, touched to the surface of the distilled water wash, and again blotted. Other acceptable methods of serum delivery are described in "Applied Microbiology," volume 21, No. 3, March 1971, pages 394–399.

(3) Dilute the microtest antigens with 0.50 percent phenolized saline and add 100 microliters (0.1 cc.) to each microplate well.

(4) Seal each plate with a plastic sealer or place unsealed in a tight incubation box as described in "Applied Microbiology," volume 23, No. 5, May 1972, pages 931–937. Incubate at 37 °C. for 18–24 hours.

(5) Read the test results as described in paragraph (f) of this section.

(e) The recommended procedure for a microagglutination test titration is as follows:

(1) Add 50 microliters (0.05cc.) of 0.85 percent physiological saline to each well of the microplate.

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[4]Information as to criteria and procedures for approval of concentrated stock suspension of stained microtest antigens may be obtained from the National Poultry Improvement Plan Staff, VS, APHIS, USDA, Presidential Building, 6525 Belcrest Road, Hyattsville, Maryland 20782.

(2) To the wells representative of the lowest dilution in the titration, add an additional 50 microliters (0.05 cc.) of 0.85 percent physiological saline making a total of 100 microliters in these wells.
(3) Transfer each serum sample as described in § 147.5(d)(2) of this section to the first well containing 100 microliters (0.10cc.) in the titration, which represents the lowest dilution.
(4) Make twofold serial dilutions of each serum by transferring 50 microliters (0.05cc.) of diluted serum from one well to the next using twelve 50 microliter microdiluters fitted in a multimicrodiluter handle. When transfers have been made to all of the wells of the desired series, the 50 microliters remaining in the microdiluters are removed by blotting, touching the microdiluters to the surface of the distilled water wash, and blotting again.
(5) Dilute the desired microtest antigen with 0.50 percent phenolized saline and add 50 microliters (0.05 cc.) to each microplate well.
(6) Seal each plate with a plastic sealer or place the unsealed microplates in a tight incubation box and incubate at 37 °C. for 18–24 hours.
(7) Read the test results as described in paragraph (f) of this section.

(f) Read the test results with the aid of a reading mirror. Results are interpreted as follows:
(1) N, or – (negative) when the microplate well has a large, distinct button of stained cells; or
(2) P, or + (positive) when the microplate well reveals no antigen button; or
(3) S, or ? (suspicious) when the microplate well has a small button. Suspicious reactions may tend to be more positive than negative [±] or vice versa [±] and can be so noted if desired.

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§ 147.6 Procedure for Determining the Status of Flocks Reacting to Tests for *Mycoplasma Gallisepticum*, *Mycoplasma Synoviae*, and *Mycoplasma Meleagridis*.

The macroagglutination tests for Mycoplasma antibodies, as described in "Standard Methods for Testing Avian Sera for the Presence of Mycoplasma Gallisepticum Antibodies" published by the Agricultural Research Service, USDA, March 1966, and the microagglutination tests, as reported in the Proceedings, Sixteenth Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians, 1973, shall be the official tests. Procedures for isolation and identification of Mycoplasma may be found in "Isolation and Identification of Avian Pathogens", published by the American Association of Avian Pathologists and §§ 147.15 and 147.16 of this part.

(a) When reactors are submitted to a laboratory as prescribed by the Official State Agency, the following criteria shall be used to determine if the flock is positive for *M. gallisepticum*, *M. synoviae*, or *M. meleagridis*:
  (1) Active air sac lesions, sinusitis, synovitis, or other clinical signs of a respiratory disease;
  (2) Recovery by culture of the Mycoplasma for which the flock was tested;
  (3) Supplemental serological test.

(b) If all of these tests are negative, the flock shall be deemed to have had no reactors for the Mycoplasma for which the flock was tested. If the Mycoplasma for which the flock was tested is isolated bacteriologically or identified as infected by a polymerase chain reaction (PCR)-based procedure approved by the Department, the flock shall be considered infected. If any of the other tests described in paragraphs (a)(1) or (3) of this section is positive, the flock shall be considered suspicious, and additional culturing procedures, and agglutination and hemagglutination inhibition (HI) tests shall be conducted according to the following sequence:
  (1) If the tube agglutination or the serum plate test is negative, the flock qualifies.
  (2) If the tube agglutination or the serum plate test is positive, the hemagglutination inhibition (HI) test and/or the serum plate dilution (SPD) test shall be conducted.
  (3) If the tube agglutination or serum plate tests are positive and HI and/ or the SPD tests are negative, the flock shall be retested in accordance with paragraph (b)(6) of this section.
  (4) If HI titers of 1:40 or SPD titers of 1:5 are found, the flock shall be considered suspicious and shall be retested in accordance with paragraph (b)(6) of this section.
  (5) If HI titers of 1:80, positive enzyme-labeled immunosorbent assay (ELISA) titers, or SPD titers of 1:10 or higher are found, in conjunction with any of the criteria described in paragraph (a)(1) of this section, the Official State Agency shall presume the flock to be infected. If the indicated titers are found, but none of the criteria described in paragraph (a)(1) of this section are evident, tracheal swabs from 30 randomly selected birds shall be taken promptly and cultured individually or a PCR-based procedure conducted on these specimens for Mycoplasma, and additional tests conducted in accordance with paragraph (b)(6) of this section before final determination of the flock status is made.
  (6) Fourteen days after the previous bleeding date, all birds or a random sample comprised of 75 birds shall be tested by the serum plate or tube agglutination test. Tested birds shall be identified by numbered bands.
  (7) If the tube agglutination test or serum plate test is negative for the Mycoplasma for which the flock was tested, the flock qualifies.
  (8) If the tube agglutination or serum plate test is positive, the HI and/ or SPD test shall be conducted on the reacting samples.
  (9) On the retest, if the tube agglutination or serum plate tests are positive at the same or higher rate and the HI or SPD tests are negative, the flock shall be considered suspicious and shall be retested in accordance with paragraph (b)(6) of this section.

(10) On the retest if HI titers of 1:80 and/or SPD titers of 1:10 or higher are found, the flock shall be considered infected: *Provided*, That, at the discretion of the Official State Agency, additional tests may be conducted in accordance with paragraph (b)(6) of this section before final determination of the flock status is made.

(11) If HI titers of 1:80 and/or SPD titers of 1:10 or higher are found on the second retest, the flock shall be considered infected for the Mycoplasma for which it was tested.

(12) If the tube agglutination or serum plate tests are found on the second retest to be positive at the same or higher rate and the HI and/or SPD tests are negative, the flock should be considered infected: *Provided*, That if the status of the flock is considered to be equivocal, the Official State Agency may examine reactors by the in vivo bio-assay, PCR-based procedures, and/or culture procedures before final determination of the flock status is made.

(13) If the in vivo bio-assay, PCR-based procedures, and culture procedures are negative, the Official State Agency may qualify the flock for the classification for which it was tested.

(14) If the in vivo bio-assay, PCR-based procedures, or culture procedures are positive, the flock shall be considered infected: *Provided*, That if only the bio-assay is positive, additional in vivo bio-assay, PCR-based procedures, or cultural examinations may be conducted by the Official State Agency before final determination of the flock status is made.

(15) If the in vivo bio-assay, PCR-based procedures, or cultures are positive on retest, the flock shall be considered infected for the Mycoplasma for which it was tested.

§ 147.7 Standard Test Procedures for Mycoplasma.[5]

The serum plate agglutination test, tube agglutination test, and the enzyme-linked immunosorbent assay (ELISA) should be considered basic screening tests for Mycoplasma antibodies. The test selected will depend on preference, laboratory facilities, and availability of antigen. These three tests, though quite accurate, determine flock status rather than individual bird status, since occasional reactions are nonspecific. Under normal circumstances, the rate of such nonspecific reactions is low. Nonspecific reactions may occasionally be high, particularly after the use of erysipelas bacterin in turkeys and where Mycoplasma antibodies are present for closely related Mycoplasma other than for the species being tested. The hemagglutination inhibition (HI) test is too cumbersome for routine screening use. Positive reactions are extremely accurate however, and are useful in evaluating serum

[5]For additional information on Mycoplasma test procedures, refer to the following references: Proc. 77th Annual Meeting, U.S. Animal Health Association, 1973; Isolation and Identification of Avian Pathogens, 3rd Edition; Methods for Examining Poultry Biologics and for Identifying and Quantifying Avian Pathogens, 1991.

samples that react with the ELISA, plate, and/or tube antigens. The test should be conducted with 4 HA units. Titers of 1:80 or greater for both chicken and turkey sera are considered positive, while a 1:40 or 1:20 titer would be strongly suspicious and additional tests should be required.

(a) Serum plate test

(1) The serum plate agglutination test for Mycoplasma is conducted by contacting and mixing 0.02 ml of test serum with 0.03 ml of serum plate antigen on a glass at room temperature. The standard procedure is:
   (i) Allow antigen and test serums to warm up to room temperature before use.
   (ii) Dispense test serums in 0.02 ml amounts with a pipette or standardized loop (rinsed between samples) to 1/2 inch squares on a ruled glass plate. Limit the number of samples (no more than 25) to be set up at one time according to the speed of the operator. Serum should not dry out before being mixed with antigen.
   (iii) Dispense 0.03 ml of antigen beside the test serum on each square. Hold antigen dispensing bottle vertically.
   (iv) Mix the serum and antigen, using a multimixing device if large numbers are to be run at one time.
   (v) Rotate the plate for 5 seconds. At the end of the first minute, rotate the plate again for 5 seconds and read 55 seconds later.

(2) A positive reaction is characterized by the formation of definite clumps, usually starting at the periphery of the mixture. Most samples that are highly positive will react well within the 2-minute test period. Reactions thereafter should be considered negative, although partial agglutination at 3 and 5 minutes may warrant further retesting. High-quality antigen contacted with negative serum will usually dry up on the plate without visible clumping. Whenever samples are run, the antigen should be tested against known positive and negative control serums. Standard reference antigens and negative and positive titered sera are available from the National Veterinary Services Laboratories (NVSL), P.O. Box 884, Ames, Iowa 50010.

(3) Since it is difficult to measure uniform amounts of serum with a calibrated loop, this technique should not be used in conducting an official test.

(b) Serum plate dilution test

(1) The serum plate dilution (SPD) test may be used to evaluate possible nonspecific reactions, gain additional information to evaluate positive plate tests occurring in an unexpected manner, and/or to evaluate the level of Mycoplasma antibodies present in the serum sample. If sufficient serum is available, the following method would provide the dilutions required to conduct the test.
   (i) Rack three tubes and put 0.8 ml of phosphate-buffered saline (PBS) in tube 1 and 0.5 ml of PBS in tubes 2 and 3.
   (ii) Pipette 0.2 ml of the test serum into tube 1 and discard the pipette.
   (iii) With a pipette, mix the serum and PBS in tube 1 and withdraw 0.5 ml and add to tube 2.
   (iv) Repeat the process in step (iii), mixing the contents of tube 2 and transferring 0.5 ml to tube 3.

(v) Conduct the test, as described for the serum plate test in paragraph (a), on the undiluted sample and on samples in tubes 1, 2, and 3 after proper mixing of each dilution.

(vi) To assist in the evaluation of the test, conduct concurrent SPD tests using both positive 1:80 and positive 1:160 HI sera for the Mycoplasma being tested. The antigen should be pretested for reactivity with standard serum at the 1:5 and 1:10 dilution.

(vii) Interpretation of the SPD test results should be based on the criteria in § 147.6(b) of this part.

(c) Tube agglutination test

(1) The Mycoplasma tube agglutination test is conducted by mixing 0.08 ml of test serum with 1.0 ml of diluted (1:20) antigen in a tube and allowing the mixture to react for 18–24 hours at 37 °C. The diluent will be the standard phosphate-buffered saline with phenol. This solution is made up as follows:

Sodium hydroxide (C.P.)	0.15 g.
Sodium chloride (C.P.)	8.50 g.
Potassium dihydrogen phospate (KH2P04)(C.P.)	0.68 g.
Phenol (Crystal) (C.P.)	2.50 g.
Distilled water to make 1,000ml	

The Ph of the buffered phenolized saline will be 7.1–7.2 if all reagents are accurately measured. The stock tube antigen is diluted 1:20 with buffered phenolized saline. The procedures for the tube test are as follows:

(i) Rack 12 x 75 mm clean tubes and identify the tubes according to the sample to be tested.

(ii) Add 0.08 ml of the individual test serum to each tube. This will create approximately a 1:12.5 screening dilution of test serum when 1.0 ml of diluted antigen is added. The use of a pipetting device will insure proper mixing of serum and antigen.

(iii) To interpret positive reactions to the 1:12.5 dilution, two additional dilutions may be made by adding 0.04 ml of serum for 1:25 dilution and 0.02 ml of serum for 1:50 dilution, with the addition of 1.0 ml of diluted antigen as indicated in paragraph (c)(1)(ii) of this section.

(iv) Shake racks and incubate test systems for 18–24 hours at 37 °C.

(2) Tests are read against a dark background under indirect fluorescent light. Regarded as a positive reaction is a clearing of the supernatant fluid, with visible sediment in the bottom of the tube. Incomplete reactions are suspect. Positive and negative control serums should be incorporated into each day's run of tests. Reactions at 1:25 or greater are considered positive. They should be confirmed by the HI test. Incubation for periods greater than 24 hours may be helpful in evaluating suspicious reactions and need for possible retesting or other diagnostic tests.

(d) Hemagglutination Inhibition (HI) test

The Mycoplasma HI test is conducted by the constant-antigen, decreasing-serum method. This method requires using a 4-hemagglutination (HA) unit of diluted antigen. Differences in the number of HA units used will change the titers of positive sera markedly. Standard HA antigens for *Mycoplasma gallisepticum*, *M. synoviae*, and *M. meleagridis* are available from NVSL. The antigen has been titrated and diluted to approximately 1:640. The HA titration of each sample should be checked as described in paragraph (d)(2) on initial use or after long storage. To maintain HA activity, the undiluted HA antigen should be stored at –60 to –70 °C. The test procedures are illustrated in Tables 2 and 3 of this paragraph.

(1) Preparation of materials

(i) Prepare phosphate-buffered saline (PBS) as follows:

Sodium hydroxide (C.P.)	0.15 g.
Sodium chloride (C.P.)	8.50 g.
Potassium dihydrogen phosphate (KH2PO4) (C.P.)	0.68 g.
Distilled water to make 1,000 ml	

The Ph of the PBS will be 7.1–7.2 if all reagents are accurately measured.

(ii) Collect the turkey or chicken red blood cells (RBC's) in Alsever's solution which has been prepared as follows:

Sodium citrate	8.0 g.
Sodium chloride	4.2 g.
Dextrose	20.5 g.
Distilled to make 1,000 ml	

The sodium citrate and sodium chloride are dissolved in 800 ml distilled water and sterilized at 15 lbs. pressure for 15 minutes. Dissolve the dextrose in 200 ml distilled water, sterilize by Seitz or other type of filtration and then add aseptically to the sterile sodium citrate and sodium chloride solution.

(iii) From a turkey(s) or chicken(s) known to be free of the Mycoplasma being tested, withdraw sufficient blood with a syringe containing Alsever's solution to give a ratio of 1 part blood to 5 parts Alsever's solution (e.g., 8 ml blood in 40 ml of Alsever's solution). Centrifuge the blood suspension at 1,000 rpm for 10 minutes and remove the Alsever's solution or supernatant with a pipette.

(iv) Wash the RBC's two times in 10 or more parts of Alsever's solution, centrifuging after each washing. Centrifugation is at 1,000 rpm for 10 minutes. The supernatant fluid is removed and the RBC deposit resuspended to give a 25 percent suspension of packed RBC's in Alsever's solution. (In testing either chicken or turkey sera, the homologous RBC system must be used; i.e., use chicken cells when testing chicken serum and turkey cells when testing turkey serum.) If this suspension is kept refrigerated, it should keep for 7 or 8 days after the blood has been collected.

(v) For the test, 1 ml of the 25 percent RBC's is added to 99 ml of buffered saline to make a 0.25 percent RBC suspension.

(2) Hemagglutination (HA) antigen titration

The HA stock antigen is stored at –70 °C in PBS buffer containing 25 percent glycerin (vol/vol) in a concentrated suspension (i.e., 320–640 HA units/ml) in screwtype vials. Under such conditions, potency will be retained for years. There will be a rapid loss of titer if improperly stored. The titer of HA antigen is determined as illustrated in Table 1 and described in subparagraphs (d)(2)(i) through (x) of this paragraph.

Table 1 Titration of Hemagglutination (HA) Antigen

	Tube No.						
Reagents (ml)	1	2	3....	8	9	10	11[a]
PBS	0.8	0.5	0.5	0.5	0.5	0.5	0.5
Antigen	0.2						
Transfer	0.5→	0.5→	0.5→	0.5→	0.5→	0.5→[c]	
0.25% RBC	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ant. dilution	1:5	1:10	1:20	1:640	1:1,280	1:2,560	
Results[b]	+	+	+	+	−	−	

[a]Tube 11, PBS/RBC control.
[b]+ = HA; −= no HA (sample titer 1:640).
[c]Discard 0.5 ml.

(i) Rack a series of 11 chemically clean 12×75 mm test tubes. Label the tubes 1–11 left to right.
(ii) Put 0.8 ml of PBS in tube 1 and 0.5 ml of PBS in each of tubes 2–11.
(iii) Add 0.2 ml of antigen to tube 1. This will make a 1:5 dilution of antigen. Discard pipette.
(iv) Mix contents of tube 1 thoroughly with a clean pipette, and transfer 0.5 ml to tube 2. This will make a 1:10 dilution of antigen in tube 2. Discard pipette.
(v) Continue making serial twofold dilutions of antigen, changing pipettes after each transfer, through tube 10. This will result in a series of twofold dilutions ranging from 1:5 to 1:2560. Discard 0.5 ml of antigen dilution from tube 10.
(vi) Add 0.5 ml of 0.25 percent RBC's to tubes 1–11. Tube 11 will serve as PBS/RBC control.
(vii) Shake the rack and incubate at room temperature until the cells in the PBS/RBC control tube have settled into a compact button at the bottom of the tube.
(viii) If turkey sera is also to be tested for HI titer, repeat steps outlined in (d)(2)(i) through (vii) of this paragraph, using 0.25 percent turkey RBC's.
(ix) The end point of the titration is the highest dilution of antigen that produces complete agglutination of the RBC's, as evidenced by the formation of a thin sheet of cells covering the concave bottom of the tube. For example, if complete agglutination is produced through tube 8 (a dilution of 1:640 of antigen), the antigen would be said to titer 640, the reciprocal of the dilution.
(x) Specificity of HA antigen should be determined by conducting HI tests with specific chicken sera of variable HI titers. Specific turkey sera of varying HI titers should be used if turkey sera is also to be tested.

Table 2 Hemagglutination Inhibition (HI) Test

	Tube No.						
Reagents (ml)	1[a]	2	3	8	9	10	11[b]
PBS	0.8	0.0	0.0	0.0	0.0	0.0	0.5
8-unit antigen	0	0.5	0.0	0.0	0.0	0.0	0.0
4-unit antigen	0	0	0.5	0.5	0.5	0.5	0.0
Test serum	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Transfer	0.5→	0.5→	0.5→	0.5→	0.5→	0.5 ↓[c]	
0.25% RBC	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Serum dilution	1:5	1:10	1:20	1:640	1:128	1:256	
				0	0		

[a]Tube 1. Serum control.
[b]Tube 11. PBS/RBC control.
[c]Discard 0.5 ml.

Table 3 Antigen Control

	Tube No.				
Reagents (ml)	1	2	3	4	5
4-unit antigen	1.0	0	0	0	0
PBS	0	0.5	0.5	0.5	0.5
Transfer	0.5→	0.5→	0.5→	0.5→	0.5 ↓[b]
0.25% RBC	0.5	0.5	0.5	0.5	0.5
Unit Antigen/tube	4	2	1	1/2	1/4
Results[a]	+	+	+	-	-

[a]+=HA; −= no HA.
[b]Discard 0.5 ml.

(3) Reagents for mycoplasma HI test

(i) Eight-unit antigen (Dilution factor for stock antigen is established by dividing titer by 8; i.e., 640 antigen is diluted 1:80 in PBS to make 8-unit antigen.)
(ii) Four-unit antigen (made by diluting surplus 8-unit antigen 1:2 with PBS).
(iii) PBS at Ph 7.0.
(iv) Unknown test serums.
(v) Positive control serum of known titer (should be from the same species as the unknown).
(vi) Negative control serum (should be from the same species as the unknown).
(vii) Solution of 0.25 percent washed RBC's.

(4) Test outline

(i) Rack 10 chemically clean 12×75 mm tubes for each serum, including controls, to be tested. Identify each row of tubes, and label tubes in each row 1–10, left to right. In row 1, add tube 11 for a PBS/RBC control.
(ii) Put 0.8 ml of PBS in tube 1 of each test row; put 0.5 ml of 8-unit antigen in tube 2 of each test row; put 0.5 ml of 4-unit antigen in each of tubes 3–10 in each test row; and put 0.5 ml of PBS in tube 11.
(iii) Add 0.2 ml of test serum to tube 1. This tube will be the serum control in the test system.
(iv) Mix and make 0.5 ml transfers from tube 1 through tube 10. This will result in serial twofold dilutions of serum starting with 1:5 and ending with 1:2560. Discard 0.5 ml from tube 10.
(v) Rack five tubes in which to set up an antigen control.
(vi) In tube 1, put 1.0 ml of 4-unit antigen; put 0.5 ml of PBS in tubes 2–5.
(vii) Make 0.5 ml serial transfers from tube 1 through tube 5, changing pipettes after each transfer. Discard 0.5 ml from tube 5. This will result in a series of tubes respectively containing 4, 2, 1, 1/2, and 1/4 units of antigen.
(viii) After 20–30 minutes at room temperature to permit antibody-antigen reaction, add 0.5 ml of 0.25 percent washed RBC's to each tube. Shake racks and incubate as for HA titration.
(ix) In this test system, positive serum should inhibit the HA activity of the antigen, while negative serum should have no effect. Inhibition will be evidenced by the formation of a free-flowing button of cells in the bottom of the tube. The titer of the serum can be calculated as the reciprocal of the highest dilution of serum that produces complete HI. Controls should read as follows:
  (A) Serum control (tube 1). Cells should settle out.
  (B) PBS/RBC control (tube 11). Cells should settle out.
  (C) Antigen control. HA in tubes 1–3. Cells should settle out in tubes 4–5.
  (D) Positive and negative serum control. Positive control should inhibit to its known titer; negative control should have no inhibitory effect.
(x) With this test system and 4 units of antigen, HI titers of 80 or above are considered positive and titers of 40 are strongly suspicious. However, titers of 10 or 20 are usually negative. Sample test results are illustrated in Table 4 in this paragraph.
(xi) If serological results from agglutination tests complemented by the HI test are inconclusive, cultural examination, bio-assay, or retesting of samples after an interval of at least 21 days may be indicated.

Table 4 Sample Results of HI Tests

	[Tube and Serum Dilution]									
	1 1:5	2 1: 10	3 1: 20	4 1: 40	5 1: 80	6 1: 160	7 1: 320	8 1: 640	9 1: 1280	10 1: 1260
Serum A (HI neg.)	–	+	+	+	+	+	+	+	+	+
Serum B (HI 1:40)	–	–	–	–	+	+	+	+	+	+
Serum C (HI 1:60)	–	–	–	–	–	–	+	+	+	+
Serum D (HI 1:20)	–	–	–	+	+	+	+	+	+	+

+, HA.
–, no HA or HI.

(e) Procedure for Mycoplasma hemagglutination inhibition test using microtiter technique

The microtiter Mycoplasma HI test was developed from the tube HI test described in § 147.7(d). Refer to these procedures for preparation of materials not listed below.

(1) **Procedure No. 1**

(i) Materials needed

(A) Microtiter equipment (minimal); i.e., microplates, microdiluters, micropipettes, go–no-go diluter delivery tester, (0.05 ml).

(B) Phosphate-buffered saline (PBS).

(C) Reagents from NVSL; i.e., HA antigen and negative and positive titered sera for the Mycoplasma to be tested.

(D) Homologous red blood cells (RBC's) suspension 0.5 percent (2 ml of 25 percent RBC's to 98 ml of PBS) obtained from birds free of the Mycoplasma to be tested. (See paragraph (d)(1)(ii) through (v) of this section for preparation of RBC's.)

(ii) Microtiter hemagglutination (HA) antigen titration

(A) Mark off two rows of 10 wells each for antigen titer (HA is done in duplicate).

(B) Mark last well in each row for cell controls.

(C) Prepare in small test tube (12×75 mm) a starting dilution of antigen by combining 0.1 ml antigen with 0.9 ml PBS. This is a 1:10 dilution.

(D) Add 0.05 ml PBS to all wells, including cell controls.

(E) Add 0.05 ml antigen (1:10 dilution) with diluters to the first well in both rows, mix thoroughly, transfer diluter to second well of each row and mix, continuing through the 10th well of each row. With mixture in diluter from last well, check diluter on go–no-go card, then place diluter in distilled water. If diluter checks out, antigen dilution will be 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120.

(F) Add 0.05 ml of 0.5 percent RBC suspension to all wells using a 0.05 ml dropper.

(G) Seal plate (if plate is to be held over 2 hours); shake and allow to stand at room temperature until cells in cell control gather in compact button. The titer is the highest dilution in which agglutination is complete. The dilution contains 1 HA unit in 0.05 ml.

(H) Prepare a dilution of antigen which contains 8 HA units in 0.05 ml. Example: if the antigen titer is 1:640, then that dilution contains 1 HA unit per 0.05 ml. Then 640/8 = 80, or a dilution of 1:80 containing 8 HA units. Or 640/4 = 160, a dilution of 1:160 containing 4 HA units per 0.05 ml.

(iii) Microtiter HI test

(A) Prepare two dilutions of antigen, one containing 8 HA units per 0.05 ml and one containing 4 HA units per 0.05 ml. The 4-unit antigen can be prepared from the 8-unit antigen by mixing with equal parts of PBS.

(B) Mark off one row of 8 wells for each test.

(C) Prepare a 1:5 dilution of each serum to be tested in a small test tube (12×75 mm): 0.1 ml serum plus 0.4 ml PBS or 0.05 ml serum plus 0.20 ml PBS.

(D) Add 0.05 ml PBS with the 0.05 ml dropper to the first well in each row.

(E) Add 0.05 ml of 8-unit antigen to well 2 in each row.

(F) Add 0.05 ml of 4-unit antigen to wells 3 through 8 for each row.

(G) For each serum to be tested, load 0.05 ml diluter with 1:5 dilution as prepared in paragraph (C) above and place in first well of row.

(H) Mix well and transfer loaded diluter to well 2. Continue serial twofold dilutions through well number 8.

(I) Well 1 (serum dilution of 1:10) is serum control. Well 2 = 1:20 dilution; well 3 = 1:40 dilution; well 4 = 1:80 dilution; well 5 = 1:160 dilution; well 6 = 1:320 dilution; well 7 = 1:640 dilution; and well 8 = 1:1280 dilution.

(J) Antigen control.

(1) Mark off 6 wells for antigen controls.

(2) Add 0.05 ml PBS to wells 2, 3, 4, 5, and 6.

(3) Add 0.05 ml 8-unit antigen to wells 1 and 2.

(4) With empty diluter, mix contents of well 2. Continue serial twofold dilutions through well 6.

(5) Well 1 contains 8 units; well 2 contains 4 units; well 3 contains 2 units; well 4 contains 1 unit; well 5 contains 1/2 unit; and well 6 contains 1/4 unit.

(6) Mark off two wells for cell controls and add 0.05 ml PBS to each.

(7) After 20–30 minutes at average room temperature (20–23 °C) to permit antibody-antigen reaction, add 0.05 ml of a 0.5 percent suspension of RBC's to all wells.

(8) Seal all wells (if wells are to be held over 2 hours). Shake the plate thoroughly.

(9) Incubate at room temperature for 30–45 minutes.

(K) Interpretation: The HI titer is the highest serum dilution exhibiting complete inhibition of hemmagglutination as indicated by flowing of cells when the plate is tilted. Serum having a titer of 1:80 or greater is considered positive. A titer of 1:40 or 1:20 is suspicious.

(2) **Procedure No. 2.** Purpose: To test for antibodies to avian mycoplasma by hemagglutination (HI). The test uses the constant antigen, titered-sera method for measuring antibodies to *M. gallisepticum, M. synoviae,* or *M. meleagridis.*

(i) Materials needed.

(A) *M. gallisepticum, M. synoviae,* and/or *M. meleagridis* HI antigens.

(B) Positive and negative control sera.

(C) Phosphate buffered saline (PBS).

(D) Microtiter plates, 96-well, U-button.

(E) 12-channel pipettor (Titerek).

(F) 50µL pipettor (Pipetman P200).

(G) Pipette tips.

(H) 0.5 percent homologous red blood cells (RBC's) in PBS (use RBC's from the same species being tested).

(I) Plate-sealing tape.

(J) Mirrored plate reader.

(ii) Microtiter hemagglutination antigen (HA) titration.

(A) Perform standard hemagglutination test (HA) on mycoplasma antigen to determine titer of antigen.

(1) Dispense 50 µL of PBS into each well of 3 rows of a 96-well microtiter plate.

(2) Dispense 50 µL of stock antigen into the wells of 2 rows.

(3) Perform serial two-fold dilutions (50 µL) using a 12-channel pipettor. The dilution series will be from 1:2 to 1:4096.

(4) Add 50 µL of 0.5 percent homologous RBC's to each well of all 3 rows. The row with no antigen serves as an RBC control.

(B) Incubate at room temperature (approximately 30 minutes) until the control RBC'c give tight buttons. The HA titer is read as the last well to give a complete lawn (hemagglutination). The desired endpoint is 4 HA units. The well containing the 1:4 dilution should give a complete HA while the 1:8 dilution should show less than complete HA.

(C) Dilute stock antigen to 4 HA units for the HI test. The dilution required to give 4 HA units is calculated by dividing the stock antigen HA titer by 8. (Example: 1:320 HA units + 8 + 40, dilute stock antigen 1:40.)

(iii) Hemagglutination inhibition assay.

(A) Label one column (A to H) of a 96-well, U-bottom microtiter plate for each sample, each positive and negative control sera, antigen backtitration, and RBC control.

(B) Add 40 µL of PBS to the top row of wells (row A) of the plate.

(C) Add 25 µL of PBS to all remaining wells of the plate.

(D) Add 10 µL of each test sera to well A of each column (making a 1:5 sera dilution).

(E) Serially dilute 25 µL from well A through H using a 12-channel pipettor. Discard the final 25 µL. Row A = 1:5...row H = 1:640.

(F) With an Oxford doser, add 25 µL of 4 HA units antigen to wells B through H. Well A serves as sera control.

(G) Prepare an antigen backtitration by adding 25 µL of PBS to each well of one column. Add 25 µL of diluted antigen to well A and serially dilute 25 µL from wells A to D. This prepares 1:2, 1:4, 1:8, and 1:16 dilutions. (It is recommended that the antigen control backtitration be performed before the diluted antigen is used in the assay. Dilution problems could be detected and corrected before the inappropriately diluted antigen is used in the assay.)

(H) Leave a column of wells blank for an RBC control.

(I) Agitate gently and incubate for 30 minutes at room temperature.

(J) Add 50 µL of 0.5 percent RBC's to all wells. Note: Do not agitate after RBC's have been added (agitation may result in false positive reactions by causing the RBC's to fall, resulting in "false" buttons).

(K) Cover the plate with sealing tape. Incubate at room temperature for 30 minutes or until control RBC's give a tight button.

(L) Read the reaction on a mirrored plate reader.

(iv) Results.

(A) The titer is reported as the reciprocal of the last dilution to give a tight button of RBC's. The final dilution scheme includes the antigen in the dilution calculation and is as follows: B=1:20, C=1:40, D-1:80, E=1:160, F=1:320, G=1:640, H=1:1,280.

(B) For the assay to be valid:

(1) The positive control sera must give a result within one dilution of the previously determined titer.

(2) The negative control sera must be negative.

(3) The backtitration of the antigen must be 1:4 or 1:8.

(4) The RBC control must give tight, non-hemolyzed buttons.

(5) Sera controls (well A of each test sera) must not have non-specific agglutination or hemolysis. If negative, report as "negative with non-specific agglutination or non-specific hemolysis" or "unable to evaluate due to non-specific agglutination or hemolysis" or treat the serum to remove the non-specific agglutination and repeat the test. (See paragraph (e)(2)(v) of this section.)

(v) Treatment to remove non-specific agglutination.

(A) Purpose. Treatment of serum to remove non-specific agglutination that is interfering with HI assays.

(B) Specimen. Serum.

(C) Materials. Homologous RBC's (chicken or turkey), 50 percent solution PBS, centrifuge, incubator, 4 ˚C (refrigerator).

(D) Procedure.

(1) Prepare a 1:5 dilution of test serum by adding 50 µL of serum to 200 µL of PBS.

(2) Prepare a 50 percent solution of RBC's by adding equal volumes of packed RBC's to PBS. Mix well.

(3) Add 25 µL of 50 percent RBC solution to the serum dilutions.

(4) Vortex gently to mix.

(5) Incubate at 4 ˚C for 1 hour.

(6) Centrifuge to pellet the RBC's.

(7) Use the supernatant to perform the HI assay. Modify the dilution scheme in the assay to consider the initial 1:5 dilution prepared in the treatment. For the 1:5 dilution scheme, do not add PBS to row A. Add 50 µL of the 1:5 treated supernatant to row A. Serially dilute 25 µL from rows A through H. This prepares a serum dilution of 1:10 through 1:60 in rows B through H.

§ 147.8 Procedures for Preparing Egg Yolk Samples for Diagnostic Tests.

The following testing provisions may be used for retaining the classification U.S. *M. Gallisepticum* Clean under § 145.23(c)(1)(ii)(C) and § 145.33(c)(1)(ii)(C), and for retaining the classification U.S. *M. Synoviae* Clean under § 145.23(e)(1)(ii)(B) and § 145.33(e)(1)(ii)(B).

(a) Under the supervision of an Authorized Agent or State Inspector, the eggs which are used in egg yolk testing must be selected from the premises where the breeding flock is located, must include a representative sample of 30 eggs collected from a single day's production from the flock, must be identified as to flock of origin and pen, and must be delivered to an authorized laboratory for preparation for diagnostic testing.

(b) The authorized laboratory must identify each egg as to the breeding flock and pen from which it originated, and maintain this identity through each of the following:
   (1) Crack the egg on the round end with a blunt instrument.
   (2) Place the contents of the egg in an open dish (or a receptacle to expose the yolk) and prick the yolk with a needle.
   (3) Using a 1 ml syringe without a needle, aspirate 0.5 ml of egg yolk from the opening in the yolk.
   (4) Dispense the yolk material in a tube. Aspirate and dispense 0.5 ml of PBS (phosphate-buffered saline) into the same tube, and place in a rack.
   (5) After all the eggs are sampled, place the rack of tubes on a vortex shaker for 30 seconds.
   (6) Centrifuge the samples at 2500 RPM (1000×g) for 30 minutes.
   (7) Test the resultant supernatant for *M. gallisepticum* and *M. synoviae* by using test procedures specified for detecting IgG antibodies set forth for testing serum in § 147.7 (for these tests the resultant supernatant would be substituted for serum); except that a single 1:20 dilution hemagglutination inhibition (HI) test may be used as a screening test in accordance with the procedures set forth in § 147.7.

Note:—For evaluating the test results of any egg yolk test, it should be remembered that a 1:2 dilution of the yolk in saline was made of the original specimen.

Subpart B—Bacteriological Examination Procedure

§ 147.10 Laboratory Procedure Recommended for the Bacteriological Examination of Egg-Type Breeding Flocks With *Salmonella enteritidis* Positive Environments.

Birds selected for bacteriological examination from egg-type breeding flocks positive for *Salmonella enteritidis* after environmental monitoring should be examined as described in § 147.11(a) of this subpart, with the following exceptions and modifications allowed due to the high number of birds required for examination:

(a) Except when visibly pathological tissues are present, direct culture, §147.11(a)(1) of this subpart, may be omitted; and

(b) Enrichment culture of organ (non-intestinal) tissues using a non-selective broth, § 147.11(a)(2) of this subpart, may be omitted.

§ 147.11 Laboratory Procedure Recommended for the Bacteriological Examination of Salmonella.

(a) For egg- and meat-type chickens, waterfowl, exhibition poultry, and game birds.

All reactors to the Pullorum-Typhoid tests, up to at least four birds, should be cultured in accordance with both *direct* (paragraph (a)(1)) and *selective enrichment* (paragraph (a)(2)) procedures described in this section. Careful aseptic technique should be used when collecting all tissue samples.

(1) Direct culture (refer to illustration 1). Grossly normal or diseased liver, heart, pericardial sac, spleen, lung, kidney, peritoneum, gallbladder, oviduct, misshapen ova or testes, inflamed or unabsorbed yolk sac, and other visibly pathological tissues where purulent, necrotic, or proliferative lesions are seen (including cysts, abscesses, hypopyon, and inflamed serosal surfaces), should be sampled for direct culture using either flamed wire loops or sterile swabs. Since some strains may not dependably survive and grow in certain selective media, inoculate *non-selective plates* in addition to two selective plating media. Refer to illustration 1 for recommended bacteriological recovery and identification procedures.[6] Proceed immediately with collection of organs and tissues for selective enrichment culture.

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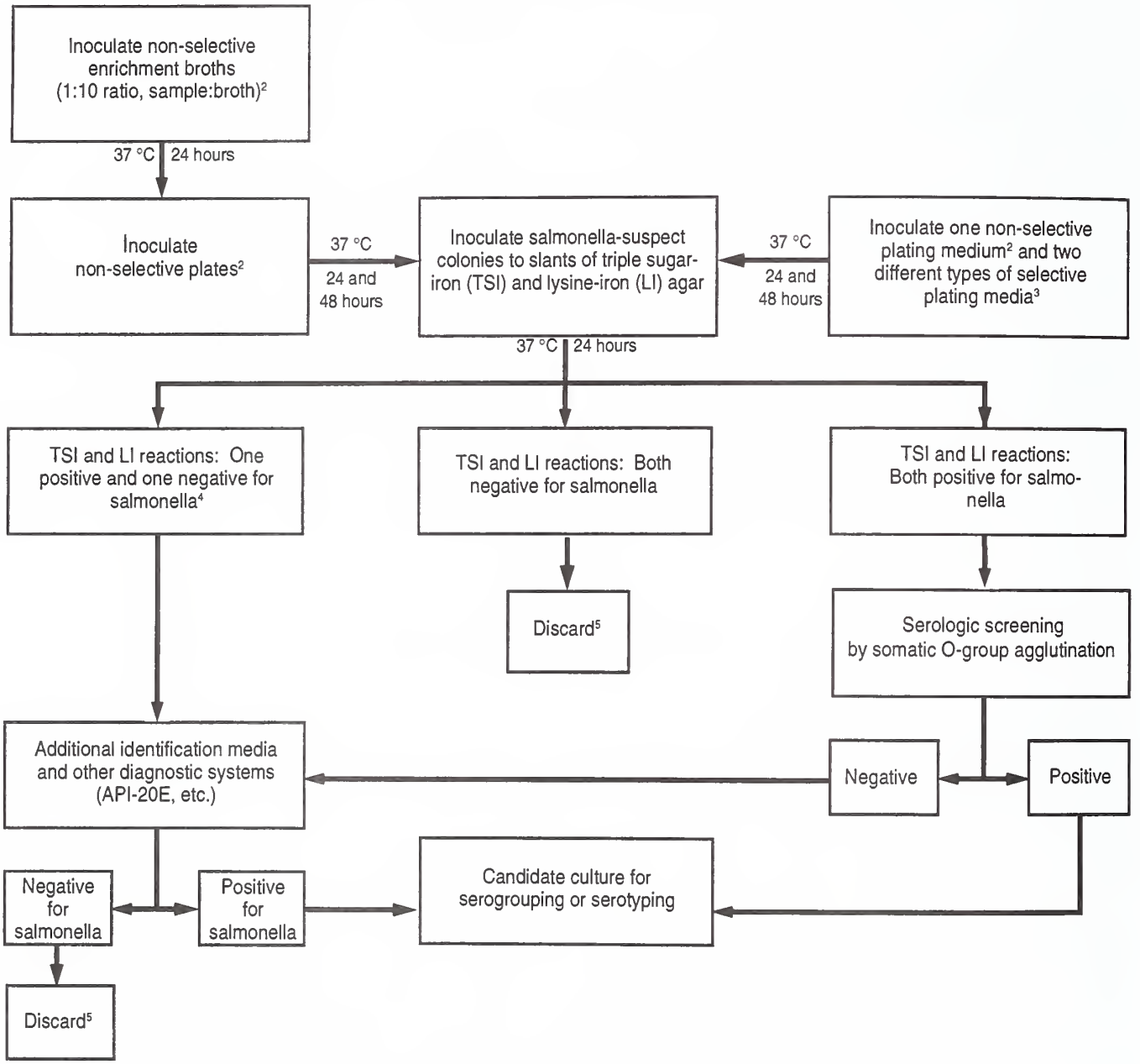
[6] Biochemical identification charts may be obtained from "A Laboratory Manual for the Isolation and Identification of Avian Pathogens," chapter 1, Salmonellosis. Third edition, 1989, American Association of Avian Pathologists, Inc., Kendall/Hunt Publishing Co., Dubuque, IA 52004-0539.

ILLUSTRATION 1—Organ (non-intestinal) tissues[1]
Pullorum-Typhoid reactors



[1]All pullorum-typhoid reactors should also be evaluated with selective enrichment broths (refer to illustration 2).
[2]Beef extract or infusion broths and plates are preferred. Comparable non-selective media may also be used.
[3]Inoculate brilliant green (BG) or BG-Novobiocin (BGN) AND another selective media such as xylose-lysine-desoxycholate (XLD) or XLD-Novobiocin (XLDN).
[4]If combined results with TSI and LI agars, additional identification media, and O-group screening procedures are inconclusive, restreak original colony onto selective plating media to check for purity.
[5]Reevaluate if epidemiologic, necropsy, or other information indicates the presence of an unusual strain of Salmonella.

(2) Selective enrichment culture (refer to illustration 2). Collect and culture organ samples separately from intestinal samples, with intestinal tissues collected last to prevent cross-contamination. Samples from the following organs or sites should be collected for culture in selective enrichment broth. A non-selective broth culture (illustration 1) of pooled organs and sites should also be included as described in paragraph (a)(3) of this section.

(i) Heart (apex, pericardial sac, and contents if present.);

(ii) Liver (portions exhibiting lesions or in grossly normal organs, the drained gallbladder and adjacent liver tissues.);

(iii) Ovary–Testes (entire inactive ovary or testes, but if ovary is active, include any atypical ova.);

(iv) Oviduct (if active, include any debris and dehydrated ova.);

(v) Kidneys and spleen; and

(vi) Other visible pathological sites where purulent, necrotic, or proliferative lesions are seen.

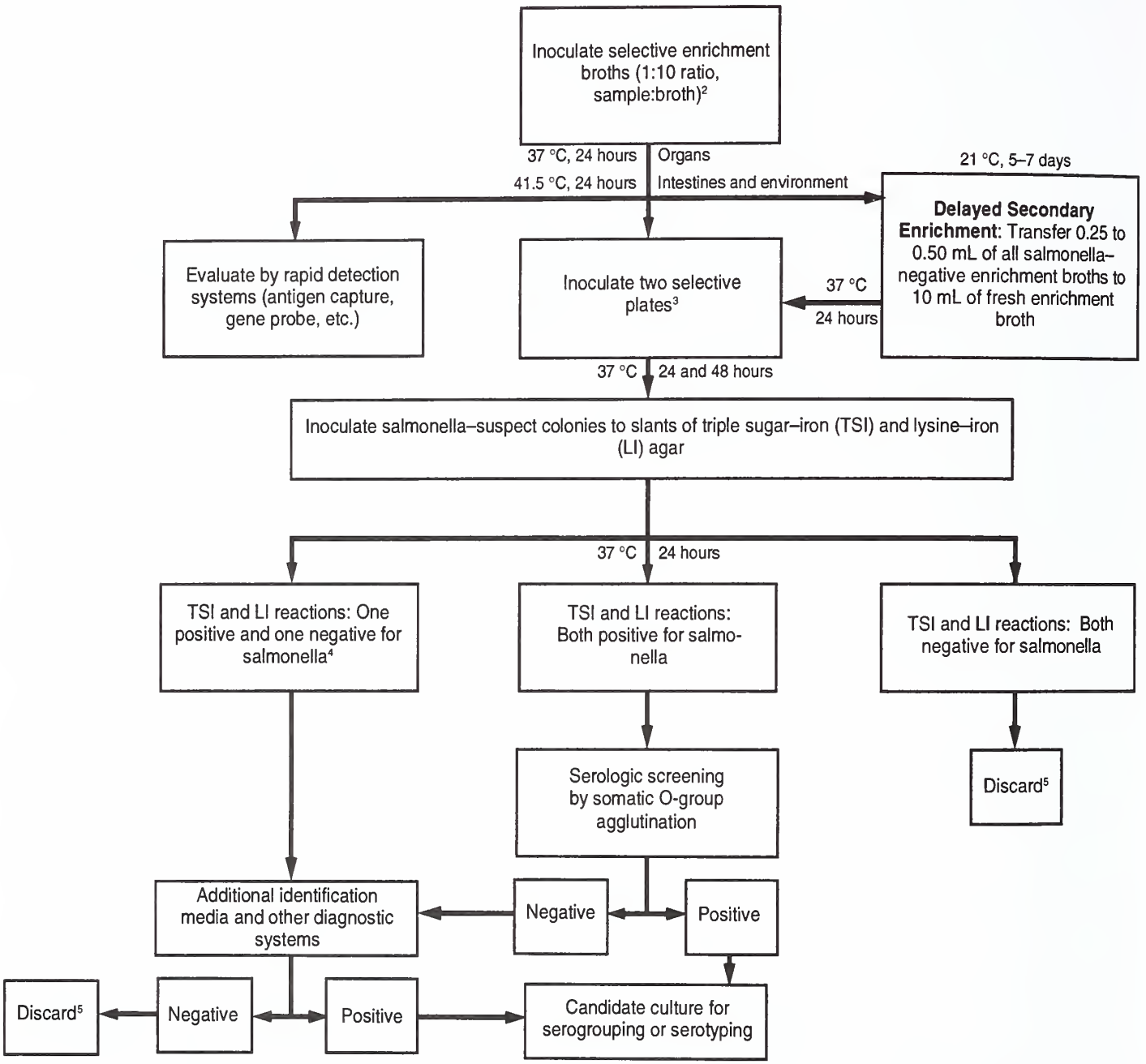
(3) From each reactor, aseptically collect 10 to 15 g, or the nearest lesser amount available, from each organ or site listed in paragraph (a)(2) of this section and mince, grind, and blend them completely in 10 times their volume of beef extract broth or a comparable non-selective broth. Organs or sites listed in paragraph (a)(2) of this section may be pooled from the same individual bird. Suspensions should be transferred in 10-ml aliquots to 100ml of both tetrathionate brilliant green (TBG) (Hajna or Mueller–Kauffman) broth and a separate non-selective broth and incubated 37 °C for 24 hours. Refer to illustration 2 for recommended bacteriological recovery and identification procedures, *including delayed secondary enrichment* and combinations of plating media that significantly suppress the overgrowth of contaminants, such as brilliant green Novobiocin (BGN) and Xylose–Lysine–Tergitol 4 (XLT4).

(4) From each reactor, make a composite sample of the following parts of grossly normal or disease tissues from the digestive tract: Crop wall, duodenum (including portions of the pancreas), jejunum (including remnant of yolk-sac attachment), both ceca, cecal tonsils, and rectum-cloaca. Aseptically collect 10–15 g or the nearest lesser amount available from each specified digestive or intestinal tissue, and mince, grind, and blend them completely in 10 times their volume of TBG broth. The digestive/intestinal tissues may be pooled from the same individual bird. Do not pool tissues from different birds. Transfer 10 ml of the described digestive TBG suspensions into 100 ml of TBG broth, and incubate at 41.5 °C for 24 hours. Cultures may be incubated at 37 °C if 41.5 °C incubators are not available. The higher incubation temperatures for TBG broth reduce populations of competitive contaminants common in gut tissue. Refer to illustration 2 for recommended bacteriological recovery and identification procedures, *including delayed secondary enrichment* and combinations of plating media that significantly suppress the overgrowth of contaminants, such as BGN and XLT4.

(5) A system such as the Analytical Profile Index for Enterobacteriaceae (API) may be utilized to aid cultural identifications.

(6) All isolates culturally identified as *salmonellae* should be serogrouped or serotyped.

ILLUSTRATION 2—Environmental, organ, and intestinal samples.[1] Environmental monitoring programs and pullorum–typhoid reactors.



[1]Organ tissues from all reactor birds should also be evaluated without selective enrichment (refer to illustration 1).
[2]Hajna TT or Mueller–Kauffmann tetrathionate enrichment broth is preferred over selenites.
[3]For enrichment broths of organ samples, inoculate xylose–lysine–desoxycholate (XLD) or XLD–Novobiocin (XLDN) and brilliant green (BG) or BG–Novobiocin (BGN) media. One of the media shall be either XLDN or BGN. For enrichment broths of intestinal or environmental samples, inoculate xylose–lysine–tergitol 4 (XLT4) or XLDN and BGN or BG media.
[4]If combined results with TSI and LI agars, additional identification media, and O–group screening procedures are inconclusive, restreak original colony onto selective plating agar to check for purity.
[5]Reevalute if epidemiologic, necropsy, or other information indicates the presence of an unusual strain of Salmonella.

(b) For Turkeys.

(1) Bacteriological examination of Salmonella reactors and necropsy specimens. Grossly normal or diseased liver, heart, pericardial sac, spleen, lung, kidney, pancreas, peritoneum, drained gallbladder, oviduct, misshapen ova, testes, inflamed or unabsorbed yolk sac, and other visibly pathological tissues where purulent, necrotic, or proliferative lesions are seen (including cysts, abscesses, hypopyon, and inflamed serosal surfaces), should be directly cultured by means of a flamed wire loop or with sterile swabs.[7] Careful aseptic technique must be utilized throughout the process of collecting tissues. Selective media should not be relied upon to deal with contaminants, since some strains may not dependably survive and grow in certain selective media. Inoculate veal infusion (VI) and brilliant green (BG) agar plates. Incubate the plates for 24 and 48 hours at 37 °C. The digestive system should always be cultured separately (see paragraph (7) of this section) after other anatomical organs and systems have been collected and cultured.

(2) Bacteriologic examination of environmental and other contaminated specimens.

(i) Culture a representative sample of the specimen in tetrathionate Hajna (TTH) selective broth (TT Mueller–Kauffmann or selenite–cystine is also acceptable) at a temperature of 41–42 °C for 24 hours.

Note: Do not use selenite–cystine if double strength skim milk is used as a preservative for the sample.

(ii) Inoculate an agar plate of brilliant green novobiocin (BGN) and an agar plate of xylose–lysine–tergitol 4 (XLT4), incubate at 37 °C for 24 hours, and retain culture tubes at room temperature for 5–7 days for possible reculturing of the negative tubes using 0.25 ml in TTH.

(iii) Inoculate Salmonella suspect colonies to slants of triple sugar–iron (TSI) and lysine–iron (LI) agar and incubate at 37 °C for 24 hours. Five colony picks per plate should be taken unless 50 percent or more of the plates have Salmonella-like colonies. In that case, the number of picks may be reduced to three per plate.

(iv) Conduct serologic screening of cultures revealing typical reactions of Salmonella on TSI and LI agar slants using somatic O-group antisera agglutination or transfer for further identification to appropriate biochemical tests such as: Dextrose, lactose, sucrose, mannitol, maltose, dulcitol, malonate, gelatin, urea broth, citrate, lysine decarboxylase, ornithine decarboxylase, methyl red and Voges–Proskauer, KCN, salicin broths, indole, and hydrogen sulfide. Motility or non-motility is demonstrated by inoculating a suitable semisolid medium. The Analytical Profile Index API 20E)[8] for Enterobacteriacea (APE) system may also be used for further identification if desired.

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[7]Culture media preparation and biochemical identification charts can be obtained from Culture Methods for the Detection of Animal Salmonellosis and Arizonosis, Committee on Salmonellosis and Arizonosis, AAVLD, 1976 Iowa State University Press, Ames, IA 50010.

[8] We use trade names solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement over other products not mentioned.

(v) Serotype all Salmonella group D cultures at the National Veterinary Services Laboratory.

(3) The following organs should be aseptically collected for culture:
   (i) Heart (apex, pericardial sac, and contents if present.);
   (ii) Liver (portions exhibiting lesions or, in grossly normal organs, the drained gallbladder and adjacent liver tissues.);
   (iii) Ovary–Testes (entire inactive ovary or testes, but if ovary is active, use own judgment and include any atypical ova.);
   (iv) Oviduct (if active, include any debris and dehydrated ova.);
   (v) Pancreas and kidneys; and
   (vi) Spleen.

(4) Aseptically collect 10–15 g or whatever lesser amount is available of each organ or site listed in paragraph (3) from each reactor, and grind or blend them completely in 10 times their volume of VI broth. Organs may be processed individually or in combinations where appropriate. Suspensions should be transferred in 10-ml aliquots to 100 ml of both VI and tetrathionate brilliant green (TBG) broth and incubated at 37 °C for 24 hours. Plate the VI broth on VI and BG agar and plate the TBG broth on BG agar and incubate at 37 °C. Examine these plates after 24 and 48 hours of incubation. The contents of the gallbladder can be cultured separately by inoculating 10 ml of VI and TBG broth with cotton swabs and incubating at 37 °C for 24 hours. Plate on BG agar and incubate at 37 °C. Examine these plates after 24 and 48 hours of incubation. If contamination with pseudomonas or proteus is a problem, make platings on BG sulfapyridine (BGS) agar.

(5) Where field samples are directly inoculated into enrichment broths and a delay of several days occurs before they reach a laboratory, or if recovery of low numbers or organisms is expected from a primary culture, a secondary enrichment culture should be prepared. Subculture a week-old primary culture by transferring 1 ml of inoculum into a fresh tube 10 ml of enrichment broth. This secondary enrichment should be incubated at 37 °C for 24 hours before plating. (See paragraph (1) of this section.) TBG broth is recommended for this procedure.

(6) Make a composite sample of the following parts of grossly normal or diseased tissues from the digestive tract: Crop wall, duodenum, jejunum (including remnant of yolk-sac attachment), both ceca, cecal tonsils, and rectum–cloaca. Aseptically collect 10–15 g of each organ or tissue, or whatever lesser amount is available, and grind or blend them completely in 10 times their volume of TBG broth. Transfer 10 ml of a composite sample of a suspension from the digestive tract into 100 ml of TBG broth, and incubate flasks at 42 °C for 24 hours. Cultures may be incubated at 37 °C if 42 °C incubators are not available. The higher incubation temperatures for TBG broth reduce populations of competitive contaminants common in gut tissue. Plate on BG agar and incubate at 37 °C. Examine the plates after 24 and 48 hours of incubation. If contamination with pseudomonas or proteus is a problem make platings on BGS agar.

(7) If preferred, individual cotton swab samples may also be taken from the upper, middle, and lower intestinal tract (including both ceca and the rectum–cloaca). Deposit swabs in 10 ml of TBG broth and incubate and plate as described in paragraph (6) of this section.
(8) Transfer suspect colonies to triple-sugar–iron (TSI) agar and lysine–iron (LI) agar and incubate at 37 °C for 24 hours.
(9) Cultures revealing typical reactions of Salmonellae on TSI and LI agar slants should be transferred to any of the following appropriate biochemical tests for final identification: Dextrose, lactose, sucrose, mannitol, maltose, dulcitol, malonate, gelatin, urea broth, citrate, lysine decarboxylase, ornithine decarboxylase, methyl red and Voges–Proskauer, KCN, salicin broths, indole, and hydrogen sulfide. Motility or non-motility is demonstrated by inoculating a suitable semisolid medium.[9] The Analytical Profile Index for Enterobacteriacae (API) system may be utilized for identification if feasible. For arizonae identification, make readings daily up to 10 days. An O-nitrophenyl-beta-D-galactopyranside (ONPG) disc may be used to identify slow lactose fermenters.[10]
(10) All salmonella cultures should be serologically typed.

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§ 147.12 Procedures for Collecting Environmental Samples and Cloacal Swabs for Bacteriological Examination.

Information concerning the pen arrangement and number of birds per pen should be obtained from the owner so that the required number of samples per pen and per flock can be determined. A means of identifying each sample by pen of origin should be provided. The vehicle transporting the personnel taking the samples should be left as far as practical from the poultry pens. Sanitary precautions, including personal cleanliness, should be observed during the sampling procedure. The hands should be carefully washed with a sanitizing soap prior to the sampling. Outer clothing, including gloves, should be changed between visits to different premises so that clean clothing is worn upon entering each premises.

The used and clean apparel should be kept separate. Boots or footwear should be cleaned and disinfected between visits to different premises. Disposable caps should be provided and discarded after use on each premises. After collection, the samples should be protected from drying, light, and excessive temperatures and delivered to the laboratory within one day. If delivery is delayed, samples should be refrigerated.

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[9]Formulation for the semisolid motility medium can be obtained from: Isolation and Identification of Avian Pathogens, American Association of Avian Pathologists, University of Pennsylvania, New Bolton Center, Kennett Square, Pennsylvania 19348-1692.

[10]ONPG discs are available from: Baltimore Biological Laboratories, Cockeysville, MD 21030.

(a) Environmental Samples

Fecal material, litter, or dust to be submitted for bacteriological examination should be collected in accordance with the procedures described in paragraphs (a)(1) or (2) of this section:

(1) Procedure for sampling in broth. Authorized laboratories will provide capped tubes 1–2 cm in diameter and 15–20 cm in length which are two-thirds full of a recently made, refrigerated, sterile enrichment broth (Selenite Brilliant Green Sulfapyridine or Tetrathionate Brilliant Green) for each sample. Sufficient tubes should be taken to the premises to provide at least one tube per pen or one tube per 500 birds, whichever is greater. At least one sterile, cotton-tipped applicator will be needed for each tube. The dry applicator is first placed or drawn through fresh manure (under roost, near water troughs, cecal droppings, or diarrhetic droppings). After this and each subsequent streaking, the cotton-tipped applicator is placed in the tube of broth and swirled to remove the collected material. The applicator is then withdrawn and is used for taking additional specimens by streaking on or through areas where defecation, trampling of feces, or settling of dust are common; i.e., on or near waterers, feeders, nests, or rafters, etc. When the volume of material collected equals approximately 10 percent of the volume of the broth (usually 10–12 streakings), the applicator is placed in the tube and the stick is broken in half. The lower or cotton-tipped half is left in the broth, and the upper half is retained for future disposal. The cap is then replaced on the inoculated tube, and the sampling procedure is continued in other areas of the pen.

(2) Procedure for sampling in dry containers. A sample of fecal material, litter, or dust is placed in a sterile, sealable container. The sample shall consist of several specimens of material taken from a representative location in the pen or house. At least 10 g (approximately a heaping tablespoonful) of material shall be collected for each sample. The specimens in each sample shall be collected with a sterile tongue depressor or similar uncontaminated instrument. The samples should vary in type and consistency. Half of the samples should be comprised of material representing defecated matter from a large portion of the flock; i.e., trampled, caked material near waterers and feeders. The minimum number of samples to be taken shall be determined by the following:

Five samples from pens or houses of up to 500 birds;
Ten samples from pens or houses of 500 to 2,500 birds;
Fifteen samples from pens or houses with more than 2,500 birds.

The composite samples above may be pooled to not less than five samples at the laboratory as long as the volume of material collected equals approximately 10 percent of the volume of the broth.

(b) Cloacal swabs

Cloacal swabs for bacteriological examination are taken from each bird in the flock or from a minimum of 500 birds in accordance with the procedure described in paragraph (a)(1) of this section.

(1) *Procedure for taking cloacal swabs.* The authorized laboratory will provide sterile capped tubes or other suitable containers and cotton-tipped applicators for use in taking the cloacal swabs. The cotton-tipped applicator is inserted into the cloaca and rectum in such a manner as to insure the collection of fecal material. The swab and adhering fecal material are then placed in the tube and the stick is

broken in half, with the upper half retained for future disposal. The cloacal swabs may be combined in the sterile tubes in multiples of five or in combinations specified by the authorized laboratory.

(c) Drag-swabs

Drag-swabs for bacteriological examination should involve the exposure of at least six unpooled pads per house to promote representative sampling and some element of quantification.

(1) *Drag-swab assembly.* Assemble drag-swab sampling sets from folded-once 3-by-3-inch sterile gauze pads secured with paper clips. Bend end wires of each paper clip slightly to catch into the swab fabric, thus securing the clips to the folded pads. Use two pads, assembled as described to make each drag-swab sampling set. Securely connect one pad through the free rounded end of the paper clip to a 2-ft (0.6 m) length of size 20 fibrous wrapping twine. Similarly connect the other pad to a 1-ft (0.3 m) length of twine. Then securely connect the free ends of both lengths of twine to a small loop tied at the end of a similar 5-ft length of twine. The resulting assembly resembles the letter Y with a 5-ft long vertical stem and two diagonal branches (one 1 ft long and the other 2 ft long), with a folded swab securely attached at the end of each branch. After assembly, place each two-pad drag-swab sampling set into a sterile bag.

(2) *Procedure for taking drag-swab.*

(i) Floor litter: The Plan participants should collect two samples as follows: Drag four 3-by-3-inch sterile gauze pads premoistened with double strength skim milk[11] over the floor litter surface for 15 min minimally. Place the gauze pads used to collect the samples in 18-oz whirl-pack bags, two pads per bag with each bag containing 5 ml of double strength skim milk. This will maintain the moistness of the sample during transport. Mark the bags with the type of sample and the house identification.

(ii) Nest-boxes. The Plan participant should collect one nest-box sample by using two 3-by-3-inch sterile gauze pads premoistened with double strength skim milk. Wipe the two gauze pads used to collect the sample over assorted locations of about 10 percent of the total nesting area. Place the gauze pads used to collect the sample in an 18-oz whirl-pack bag containing 5 ml of double strength skim milk. Mark the bag with the type of sample and the house identification.

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[11] Obtain procedure for preparing double strength skim milk from USDA-APHIS "Recommended Sample Collection Methods for Environmental Samples" available from the National Poultry Improvement Plan Staff, VS, APHIS, USDA, Presidential Building, 6525 Belcrest Road, Hyattsville, Maryland 20782.

§ 147.13 Procedure for Bacteriological Culturing of Eggshells for Colon Bacilli Organisms.

Proper precautions to avoid environmental contamination of the samples during the collection and laboratory process, and proper handling of the samples following collection are essential. Each State Inspector involved in eggshell culture activities must receive instruction in the necessary sanitation procedures, sampling procedures, and sample handling by the authorized laboratory involved. The Official State Agency will maintain a record showing that the required instruction was given to each State Inspector.

(a) Sample selection

Forty (40) eggs in the top flats of each of three randomly selected cases of sanitized eggs from each flock will be utilized for each sampling.

(b) Swab procedure

A 2.5 centimeter diameter circular area of the large end of each of the eggs will be rubbed with a sterile swab previously moistened with sterile lactose broth, or other suitable liquid media provided by the authorized laboratory. One swab will be used for five eggs, and four swabs will be pooled to each sterile, capped tube provided by the authorized laboratory.

(1) From the tube containing four swabs and lactose broth or other suitable media, 1 ml will be transferred to 10 ml lactose in a fermentation tube.
(2) Incubate at 37 °C for 48 hours. The presence of acid, and gas in the amount of 10 percent or more after 24 and 48 hours of incubation, provides a presumptive conclusion of the presence of colon bacilli organisms.

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§ 147.14 Procedures to Determine Status and Effectiveness of Sanitation Monitored Program.

The following monitoring procedures[12] may be applied at the discretion of the Official State Agency:

(a) Monitor effectiveness of sanitation program

(1) Culture the surface of cased eggs periodically for fecal contaminating organisms as described in § 147.13.
(2) Culture a sample of dead-in-shell eggs periodically from each breeding flock for coliforms. The culture media will be designed to include detection of Salmonella

[12]Laboratory procedures for monitoring operations proposed here are described in the following two publications: Isolation and Identification of Avian Pathogens, American Association of Avian Pathologists, University of Pennsylvania, New Bolton Center, Kennett Square, Pennsylvania 19348-1692, 1980, and Culture Methods for the Detection of Animal Salmonellosis and Arizonosis, Iowa State University Press, Ames, Iowa 50010, 1976.

species. Such eggs should also be cultured for the dependable recovery of *Salmonella*. Culturing for the dependable recovery of *Salmonellae* should include the use of:

(i) Preenrichment broths supplemented with 35 mg ferrous sulfate per 1,000 ml preenrichment to block iron-binding, *Salmonella*-inhibiting effects of egg conalbumin; and

(ii) Tetrathionate selective enrichment broths, competitor-controlling plating media (XLT4, BGN, etc.), and delayed secondary enrichment procedures detailed in illustration 2 of § 147.11(a) of this part.

§ 147.15 Laboratory Procedure Recommended for the Bacteriological Examination of Mycoplasma Reactors.[13]

(a) Turbinates, trachea, air sacs, sinuses, nasal passages, respiratory exudates, synovial fluid, eggs (including yolk, yolk sacs, membranes and allantoic fluid), should be directly sampled with sterile swabs.

Aseptic techniques are very important as some organisms may not be suppressed by the antimicrobial agents used in this procedure. Tissue suspensions from large volumes are sometimes desirable from the sites listed above and occasionally from the oviduct and cloaca. Tissues should be ground or blended completely in 10 times their volume of Mycoplasma Broth Medium (MBM). (See paragraph (f) of this section.) Specimens submitted to referral laboratories in order of preference for recovery of the Mycoplasma organisms are: (1) live birds, (2) refrigerated fresh tissues, (3) tissue specimens packed with dry ice.

(b) Inoculate 5–10 ml of MBM with a swab, wire loop or 0.1 ml of the tissue suspension.

When evidence of growth is observed (lowered Ph or turbidity of broth) transfer each broth culture as needed to maintain the original isolates. Incubate tubes at 37 °C for at least 21 days before discarding as negative. When growth is first observed or if no growth occurs by the 4th or 5th day of incubation, inoculate broth culture onto a plate of Mycoplasma Agar Medium (MAM). (See paragraph (g) of this section.) Several cultures may be inoculated on one plate by using a wire loop or a cotton swab. Incubate plates 3–5 days at 37 °C in a high humidity chamber. If preferred, 5 percent CO2 may be added or a candle jar may be used. Tiny circular and translucent colonies with elevated centers are very suggestive of Mycoplasma. Indirect lighting and a low power or dissecting microscope are recommended for observation of the colonies as they are rarely more than 0.2–0.3 mm in diameter.

(c) Isolates must be serotyped.

(1) Isolates may be shipped in MBM with ice packs if shipment will be in transit less than 2–3 days. Longer shipments require freezing of the MBM with dry ice, or shipping MAM slants at room temperature. Isolates must have indications of growth before shipment is made.

(2) Isolates may be stored in MBM at –20 °C for 2–3 weeks, or they may be stored at –68 °C for several years.

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[13]Yoder, H. W., Jr., "Mycoplasmosis." In: Isolation and Identification of Avian Pathogens. (Stephen B. Hitchner, Chairman, Charles H. Domermuth, H. Graham Purchase, James E. Williams.) 1980, pp. 40–42, Creative Printing Company, Inc., Endwell, NY 13760.

(d) Alternate method of culture: An overlay enrichment culture for fastidious and sensitive Mycoplasma, especially for *M. meleagridis* should be included.

(1) Pour 2–3 ml of MAM into a test tube and tilt the tube until a slant approximately 45 °C is obtained. Other containers are acceptable.
(2) Overlay the slant with sufficient MBM, so that the media (including inoculum) covers the agar slope.
(3) Inoculate the culture as indicated in paragraph (b) of this section.
(4) Incubate and examine the overlay as indicated in paragraph (b) of this section.

(e) Preparation of media components[14]

(1) Deionized distilled water suitable for cell culture fluids should be used.
(2) All glassware should be carefully washed with a nonresidue detergent such as Alcojet and rinsed three times in tap water and twice in deionized distilled water.[15]
(3) Thallium acetate in a 10 percent solution is added to an approximate final concentration of 1:4000; however, highly contaminated specimens may require a final concentration of 1:2000.[16] Thallium acetate is added to deionized distilled water first, except as noted in paragraph (e)(4) of this section, to prevent the precipitation of proteins.
(4) Mycoplasma Broth Base, dextrose, phenol red, and cysteine hydrochloride are added to deionized distilled water first if autoclave sterilization is used.[17] Thallium acetate and then the remaining components are added aseptically after cooling the autoclaved media to 45 °C or less.
(5) Use sterile deionized distilled water to reconstitute penicillin.
(6) Sterile serum should be inactivated by heating at 56 °C for 30 minutes. Swine serum may be used for *M. gallisepticum*, *M. synoviae*, *M. gallinarum*, and *M. meleagridis* isolation; however, horse serum is usually recommended for *M. meleagridis* isolation.
(7) Phenol red should be prepared as a 1 percent solution.
(8) NAD (beta nicotinamide adenine dinucleotide or coenzyme I) should be prepared as a 1 percent solution.[18]
(9) Cysteine hydrochloride, prepared as a 1 percent solution, is used to reduce the NAD for *M. synoviae* growth.
(10) A purified agar product such as Nobel (Special agar) is used in the MA*M.*[19]
(11) Adjust the Ph with NaOH.
(12) Sterilization may be accomplished by two methods:
  (i) Filtration sterilization through a 0.20 micron filter is the recommended method. Aseptic techniques must be utilized.
  (ii) Autoclave sterilization at 120 °C, 15 pounds pressure (103 kPa), for 15 minutes may be used, if preferred, when following the procedure described in paragraph (e)(4) of this section.

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[14]Trade names are used in these procedures solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement over other products not mentioned.
[15]Alcojet is available from: Alconox, Inc., New York, NY 10003.
[16]Thallium acetate may be obtained from Fischer Scientific Company.
[17]Mycoplasma Broth Base may be obtained from: (a) Product NZM 33600, Gibco Diagnostics, 2801 Industrial Drive, Madison, WI 53711. (b) Product NZ3900-3212, Scott Laboratories, Inc., 8 Westchester Plaza, Elmsford, NY 10523.
[18]NAD Grade III may be obtained from: Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178.
[19]Noble Agar may be obtained from: Difco Laboratories, Box 1058-A, Detroit, MI 48201.

(13) Phenol red, dextrose, and NAD may be omitted when culturing for *M. meleagridis* and *M. gallinarum*.
(14) When culturing for *M. meleagridis* from contaminated samples include 100 units/ml of Polymyxin B in MB*M*.

(f) Mycoplasma Broth Medium (Frey) is prepared as follows:

To 850–880 ml of deionized distilled water;
Add:

Thallium acetate (ml)	2.5 (1:4000)
Potentially contaminated samples (ml)	5.0 (1:2000)
Mycoplasma Broth Base (g)	22.5
Aqueous penicillin (units)	500,000
Sterile serum (ml)	120 to 150.0
Phenol red plus (ml)	2.5
NAD (ml)	12.5
Cysteine hydrochloride (ml)	12.5
Dextrose (g)	1.0–1.5

Adjust Ph to 7.8
Filter sterilize

(1) Broth may be stored at 4 °C for at least 2 weeks or at –40 °C for longer periods.

(g) Mycoplasma Agar Medium (Frey) is prepared as follows:

To 850–880 ml of deionized distilled water,
Add:

Mycoplasma Broth Base (g)	22.5

Adjust Ph to 7.8

Purified agar (g)	12.0

Autoclave and cool in 45 °C water bath

Thallium acetate (ml)	2.0 (1:4000)
Sterile serum at 45 °C (ml)	150.0
Aqueous penicillin (units)	400,000
NAD (ml)	12.5
Cysteine hydrochloride (ml)	12.5

(1) Rotate flask gently and pour about 15 ml of media into each petri dish.
(2) Stack petri dishes only 2–3 high in a 37 °C incubator up to 2 hours to remove excess moisture.
(3) Wrap inverted plates in sealed bundles and store at 4 °C for not more than 15 days.

(h) New component or media batches should be monitored to compensate for changes in formulation due to alterations of purity, concentration, preparation, etc.

A known series of titrations from a single culture should be made on both new and old media. The media should be compared on the basis of growth, colony size, and numbers of colonies which develop.[20]

[20]"Laboratory Procedures and Medium For The Isolation Of Mycoplasma From Clinical Materials." *Laboratory Diagnosis of Mycoplasma in Food Animals*, Proceedings of Nineteenth Annual Meeting, The American Association of Veterinary Laboratory Diagnosticians, 1976, pp. 106–115, AAVLD, 6101 Mineral Point Road, Madison, WI 53705.

§ 147.16 Procedure for the Evaluation of Mycoplasma Reactors by in Vivo Bio-assay (enrichment).

This procedure has been shown to be sensitive enough to detect less than 100 Mycoplasma organisms under proper conditions.[21] Proper conditions are defined in this section.

(a) Obtain chickens or turkeys (test birds) which are at least 3 weeks of age and are free of *M. gallisepticum*, *M. synoviae*, and *M. meleagridis* and transport them in a manner to prevent their being contaminated by any infectious avian disease.
  (1) Maintain test birds in an area that has been effectively cleaned and disinfected.
  (2) The area should be isolated from other birds or animals.
  (3) Personnel caring for the test birds should take the necessary precautions (see § 147.26(b)) to prevent the mechanical transfer of infectious avian diseases from other sources.

(b) Test birds to be used for inoculation with contaminated tissues should be serologically negative by the serum plate agglutination test.
  (1) Inoculated test birds should be isolated from non-inoculated control birds for the length of any experiment.

(c) Aseptically obtain tracheal, turbinate, and sinus mucosa, lung and sinus exudates, cervical, thoracic, and abdominal airsac tissues (including lesions), and portions of oviduct and synovial fluid from at least four suspect, donor birds. In a sterile device, blend the tissues completely in four times their volume of Mycoplasma Broth Medium (Frey), (see § 147.15(f)). Suspensions may be made from tissue pools. Inoculate test birds within 30 minutes of preparation of suspensions.
  (1) Inoculate at least four test birds for each suspension pool via the abdominal air sac and infraorbital sinus, with up to 1/2 ml of inoculum per site.
  (2) Test birds should be bled every 7 days for 35 days to identify sero-converters.
  (3) At 35 days, test birds should be sacrificed and bacteriologic isolation and identification of Mycoplasma attempted (see § 147.15). Note especially the sites of inoculation for typical gross or microscopic Mycoplasma lesions.

(d) Donor birds are considered infected when:
  (1) Test birds have serum plate antibodies for the Mycoplasma for which the donor birds were tested, regardless of HI test results, *and* control birds stay serologically negative; or
  (2) Mycoplasma organisms are isolated from the test birds and serotyped positive for the Mycoplasma for which the donor birds were tested, and control birds stay serologically *and* culturally negative.

(e) Laboratory findings may be verified by direct cultures of material from sick birds or by inoculating seronegative birds from the suspect flock and comparing serological findings with those from the test birds.

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[21]Research results are described in the following two publications: (a) Bigland, C. H. and A. J. DaMassa, "A Bio-Assay for *Mycoplasma gallisepticum*." In: United States Livestock Sanitary Association Proceedings, 67th, 1963, pp. 541–549. (b) McMartin, D. A., "*Mycoplasma gallisepticum* in the Respiratory Tract of the Fowl." In: The Veterinary Record, September 23, 1967, pp. 317–320.

§ 147.21 Flock Sanitation.

To aid in the maintenance of healthy flocks, the following procedures should be practiced:

(a) Baby poultry should be started in a clean brooder house and maintained in constant isolation from older birds and other animals. Personnel that are in contact with older birds and other animals should take precautions, including disinfection of footwear and change of outer clothing, to prevent the introduction of infection through droppings that may adhere to the shoes, clothing, or hands (see § 147.24(a)).

(b) Range used for growing young stock should not have been used for poultry the preceding year. Where broods of different ages must be kept on the same farm, there should be complete depopulation of brooder houses and other premises following infection of such premises by any contagious disease.

(c) Poultry houses should be screened and proofed against free-flying birds. An active rodent eradication campaign is an essential part of the general sanitation program. The area adjacent to the poultry house should be kept free from accumulated manure, rubbish, and unnecessary equipment. Dogs, cats, sheep, cattle, horses, and swine should never have access to poultry operations. Visitors should not be admitted to poultry areas, and authorized personnel should take the necessary precautions to prevent the introduction of disease.

(d) Poultry houses and equipment should be thoroughly cleaned and disinfected prior to use for a new lot of birds (see § 147.24(a)). Feed and water containers should be situated where they cannot be contaminated by droppings and should be frequently cleaned and disinfected. Dropping boards or pits should be constructed so birds do not have access to the droppings.

(e) Replacement breeders shall be housed at the proper density consistent with the type of building and locality and which will allow the litter to be maintained in a dry condition. Frequent stirring of the litter may be necessary to reduce excess moisture and prevent surface accumulation of droppings. Slat or wire floors should be constructed so as to permit free passage of droppings and to prevent the birds from coming in contact with the droppings. Nesting areas should be kept clean and, where appropriate, filled with clean nesting material.

(f) When an outbreak of disease occurs in a flock, dead or sick birds should be taken, by private carrier, to a diagnostic laboratory for complete examination. All Salmonella cultures isolated should be typed serologically, and complete records maintained by the laboratory as to types recovered from each flock within an area. Records on isolations and serological types should be made available to Official State Agencies or other animal disease control regulatory agencies in the respective States for followup of foci of infection. Such information is necessary for the development of an effective Salmonella control program.

(g) Introduction of started or mature birds should be avoided to reduce the possible hazard of introducing infectious diseases. If birds are to be introduced, the health status of both the flock and introduced birds should be evaluated.

(h) In rearing broiler or replacement stock, a sound and adequate immunization program should be adopted. Since different geographic areas may require certain

specific recommendations, the program recommended by the State experiment station or other State agencies should be followed.

(i) Feed, pelleted by heat process, should be fed to all age groups. Proper feed pelleting procedures can destroy many disease producing organisms contaminating feedstuffs.

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§ 147.22 Hatching Egg Sanitation.

Hatching eggs should be collected from the nests at frequent intervals and, to aid in the prevention of contamination with disease causing organisms, the following practices should be observed:

(a) Cleaned and disinfected containers should be used in collecting the eggs, and precautions taken to prevent contamination from organisms that may be present on the hands or clothing of the person making the collection.

(b) Dirty eggs should not be used for hatching purposes and should be collected in a separate container from hatching eggs. Slightly soiled eggs may be dry cleaned by hand or motor driven buffer.

(c) The visibly clean eggs should be fumigated (see § 147.25 of this chapter) or sanitized as soon as possible after collection. The sanitized eggs shall be stored in a cool place at temperatures which will prevent the eggs from sweating at any time.

(d) Egg handlers should thoroughly wash their hands with soap and water and change to clean outer garments prior to handling the sanitized eggs. Sanitized eggs should be immediately removed from the cleaning and grading area and preferably removed to a separate clean and sanitized room. A wall-installed fumigation cabinet (or authorized sanitizing equipment) through which eggs can be passed from the receiving and cleaning area to the sanitary packing and storage areas is a good practice.

(e) The sanitized eggs should be placed in new flats or sanitized reusable flats or racks. New or clean, fumigated, or otherwise sanitized used cases should be utilized for packing. Proper temperature and humidity in the egg cleaning, packing, and storage areas should be maintained. Eggs should be stored no longer than necessary before setting.

(f) The entire egg processing area should be cleaned and sanitized daily on a routine basis; dust, insects, feathers, and other airborne debris should be effectively controlled to prevent recontamination of sanitized eggs. Ink stamps and pads shall be maintained in a sterile condition.

(g) The egg processing building or area should be designed, located, and constructed of such materials as to assure that proper egg sanitation procedures can be carried out, and that the building itself can be easily, effectively, and routinely sanitized. The egg processing building or area should be considered part of a hatchery and the same construction details and physical and personnel sanitation requirements implemented.

§ 147.23 Hatchery Sanitation.

An effective program for the prevention and control of Salmonella and other infections should include the following measures:

(a) The hatchery building should be arranged so that separate rooms, with separate ventilation, are provided for each of the four operations: Egg receiving, incubation and hatching, holding of baby poultry, and disposal of offal and cleaning of trays. These rooms should be placed under isolation so that admission is granted only to specifically authorized personnel who have taken proper precautions to prevent introduction of disease.

(b) The hatchery rooms, and tables, racks, and other equipment in them should be thoroughly cleaned and disinfected frequently. All hatchery wastes and offal should be burned or otherwise properly disposed of, and the containers used to remove such materials should be cleaned and sterilized after each use.

(c) The hatching compartments of incubators, including the hatching trays, should be thoroughly cleaned and fumigated or otherwise sanitized after each hatch.

(d) Only clean eggs should be used for hatching purposes. All eggs set should be fumigated or otherwise sanitized prior to setting or as soon as possible (preferably within 12 hours) after they are placed in the incubator. They should also be fumigated or otherwise sanitized after transfer to a separate hatcher (see § 147.25).

(e) Only new or clean, fumigated, or otherwise sanitized egg cases should be used for transportation of hatching eggs. Soiled egg case fillers should be destroyed.

(f) Day-old chicks, poults, or other newly hatched poultry should be distributed in clean, new boxes. All crates and vehicles used for transporting started or adult birds should be cleaned and disinfected after each use.

§ 147.24 Cleaning and Disinfecting.

The following procedures are recommended:

(a) In the poultry houses, hatchery rooms, and delivery trucks:

(1) Settle dust by spraying lightly with the disinfectant to be used.

(2) Remove all litter and droppings to an isolated area where there is no opportunity for dissemination of any infectious disease organisms that may be present. Housing where poultry infected with a mycoplasmal disease were kept should remain closed for 7 days before removal of the litter.

(3) Scrub the walls, floors, and equipment with a hot, soapy water solution. Rinse to remove soap.

(4) Spray with a disinfectant which is registered by the Environmental Protection Agency as germicidal, fungicidal, pseudomonocidal, and tuberculocidal, in accordance with the specifications for use, as shown on the label of such disinfectant.

(b) In the hatchers:

(1) Remove trays and all controls and fans for separate cleaning. The ceiling, walls, and floors should be thoroughly wetted with a stream of water; then scrubbed with a hard bristle brush. Rinse until there is no longer any deposit on the walls, particularly near the fan opening.
(2) Replace the cleaned fans and controls. Replace the trays, preferably still wet from cleaning, and bring the incubator to normal operating temperature.
(3) The hatcher should be fumigated (see § 147.25 of this chapter) or otherwise sanitized prior to the transfer of the eggs.

(c) If the same machine is used for incubating and hatching, the entire machine should be cleaned after each hatch.

A vacuum cleaner should be used to remove dust and down from the egg trays; then the entire machine should be vacuumed, mopped, and fumigated (see § 147.25 of this chapter) or otherwise sanitized.

§ 147.25 Fumigation.

Fumigation may be used for sanitizing eggs and hatchery equipment as an essential part of a sanitation program. APHIS disclaims any liability in the use of formaldehyde for failure on the part of the user to adhere to the Occupational Safety and Health Administration (OSHA) standards for formaldehyde fumigation, published in the Dec. 4, 1987, *Federal Register* (52 FR 46168, Docket Nos. H-225, 225A, and 225B).

§ 147.26 Procedures for Establishing Isolation and Maintaining Sanitation and Good Management Practices for the Control of Salmonella and Mycoplasma Infections.

(a) The following procedures are required for participation under the U.S. Sanitation Monitored, U.S. M. Gallisepticum Clean, and U.S. M. Synoviae Clean classifications:

(1) Allow no visitors except under controlled conditions which insure sanitation. Such conditions shall be approved by the Official State Agency and the Service;
(2) Maintain breeder flocks on farms free from market birds, or follow proper isolation procedures as approved by the Official State Agency;
(3) Eliminate other domesticated fowl from breeder farm;
(4) Dispose of all dead birds by burning, deep burial, or by putting them into special disposal pits.

(b) Recommended procedures:

(1) Avoid the introduction of Salmonella, *Mycoplasma gallisepticum*, or *Mycoplasma synoviae* infected poultry;
(2) Prevent indirect transmission from outside sources through contaminated equipment, footwear, clothing, vehicles, or other mechanical means;
(3) Provide adequate isolation of breeder flocks to avoid airborne transmission from infected flocks;

(4) Minimize contact of breeder flocks with free-flying birds;
(5) Keep the rodent population and other pests under control;
(6) Tailor vaccination programs to needs of farm and area;
(7) Clean and disinfect equipment after each use;
(8) Provide clean footwear and provide an adequate security program;
(9) Clean and disinfect houses before introducing a new flock;
(10) Use well-drained range;
(11) Use clean, dry litter free of mold;
(12) Keep accurate records of death losses;
(13) Seek services of veterinary diagnostician if unaccountable mortality or signs of disease occur;
(14) Adopt and maintain a clean-egg program; and
(15) Use only crates and vehicles that have been cleaned and disinfected in accordance with the provisions of § 147.24(a) to haul live poultry to and from the premises.

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§ 147.27 Procedures Recommended to Prevent the Spread of Disease by Artificial Insemination of Turkeys.

(a) The vehicle transporting the insemination crew should be left as far as practical from the turkey pens.
(b) The personnel of the insemination crew should observe personal cleanliness, including the following sanitary procedures:
  (1) Outer clothing should be changed between visits to different premises so that clean clothing is worn upon entering each premises. The used apparel should be kept separate until laundered. This also applies to gloves worn while handling turkeys;
  (2) Boots or footwear should be cleaned and disinfected between visits to different premises;
  (3) Disposable caps should be provided and discarded after use on each premises.
(c) The use of individual straw or similar technique is highly recommended. Insemination equipment which is to be reused should be cleaned and disinfected before reusing. Equipment used for the convenience of the workers should not be moved from premises to premises.
(d) No obviously diseased flock should be inseminated. If evidence of active disease is noted after insemination is begun, operations should be stopped and the hatchery notified.
(e) Care should be taken during the collection of semen to prevent fecal contamination. If fecal material is present, it should be removed before the semen is collected. Likewise, care should be taken not to introduce fecal material into the oviduct of the hen.

Subpart E—Procedure for Changing National Poultry Improvement Plan

§ 147.41 Definitions.

Except where the context otherwise requires, for the purposes of this subpart the following terms shall be construed, respectively, to mean:

Department The U.S. Department of Agriculture.

Egg-type chickens Chickens bred for the primary purpose of producing eggs for human consumption.

Exhibition poultry Domesticated fowl which are bred for the combined purposes of meat or egg production and competitive showing.

Game birds Domesticated fowl, such as pheasants, partridge, quail, grouse, and guineas, but not doves and pigeons.

Meat-type chickens Chickens bred for the primary purpose of producing meat.

Plan Conference A meeting convened for the purpose of recommending changes in the provisions of the Plan.

Plan or NPIP The National Poultry Improvement Plan.

Service The Animal and Plant Health Inspection Service, Veterinary Services, of the Department.

State Any State, the District of Columbia, or Puerto Rico.

Waterfowl Domesticated fowl that normally swim, such as ducks and geese.

[36 FR 23121, Dec. 3, 1971, as amended at 38 FR 3038, Feb. 1, 1973. Redesignated at 44 FR 61586, Oct. 26, 1979]

§ 147.42 General.

Changes in this subchapter shall be made in accordance with the procedure described in this subpart: *Provided*, That the Department reserves the right to make changes in this subchapter without observance of such procedure when such action is deemed necessary in the public interest.

§ 147.43 General Conference Committee.

(a) The General Conference Committee shall consist of the Assistant Secretary of Agriculture for Marketing and Inspection Services, or his/her designee, one member-at-large who is a participant in the National Poultry Improvement Plan and who shall be designated as vice chairperson, and one member to be elected, as provided in paragraph (b) of this section, from each of the following regions:
  (1) North Atlantic: Maine, New Hampshire, Vermont, Massachusetts, Rhode Island, Connecticut, New York, New Jersey, and Pennsylvania.
  (2) East North Central: Ohio, Indiana, Illinois, Michigan, and Wisconsin.
  (3) West North Central: Minnesota, Iowa, Missouri, North Dakota, South Dakota, Nebraska, and Kansas.
  (4) South Atlantic: Delaware, District of Columbia, Maryland, Virginia, West Virginia, North Carolina, South Carolina, Georgia, Florida, and Puerto Rico.
  (5) South Central: Kentucky, Tennessee, Alabama, Mississippi, Arkansas, Louisiana, Oklahoma, and Texas.
  (6) Western: Montana, Idaho, Wyoming, Colorado, New Mexico, Arizona, Utah, Nevada, Washington, Oregon, California, Alaska, and Hawaii.

(b) The regional committee members and their alternates will be elected by the official delegates of the respective regions and the member-at-large will be elected by all official delegates. There shall be at least two nominees for each position, and the voting shall be by secret ballot.

(c) Three regional members shall be elected at each Plan Conference. All members shall serve for a period of 4 years, subject to the continuation of the Committee by the Secretary of Agriculture, and may not succeed themselves: *Provided*, That an alternate member who assumed a Committee member vacancy following mid-term would be eligible for re-election to a full term. When there is a vacancy for the member-at-large position, the General Conference Committee shall make an interim appointment and the appointee shall serve until the next Plan Conference at which time an election will be held. If a vacancy occurs due to both a regional member and alternate being unable to serve, the vacant position will be filled by an election at the earliest regularly scheduled national or regional Plan Conference, where members of the affected region have assembled.

(d) The duties and functions of the General Conference Committee shall be as follows:
  (1) Assist the Department in planning, organizing, and conducting the biennial National Poultry Improvement Plan Conference.
  (2) Recommend whether new proposals (i.e., proposals that have not been submitted as provided in § 147.44) should be considered by the delegates to the Plan Conference.
  (3) During the interim between Plan Conferences, represent the cooperating States in:
    (i) Advising the Department with respect to administrative procedures and interpretations of the Plan provisions as contained in 9 CFR.
    (ii) Assisting the Department in evaluating comments received from interested persons concerning proposed amendments to the Plan provisions.

(iii) Recommending to the Secretary of Agriculture any changes in the provisions of the Plan as may be necessitated by unforeseen conditions when postponement until the next Plan Conference would seriously impair the operation of the program. Such recommendations shall remain in effect only until confirmed or rejected by the next Plan Conference, or until rescinded by the committee.

(4) Serve as a forum for the study of problems relating to poultry health and as the need arises, to make specific recommendations to the Secretary of Agriculture concerning ways in which the Department may assist the industry in solving these problems.

§ 147.44 Submitting, Compiling, and Distributing Proposed Changes.

(a) Changes in this subchapter may be proposed by any participant, Official State Agency, the Department, or other interested person or industry organization.

(b) Except as provided in § 147.43(d)(2), proposed changes shall be submitted in writing so as to reach the Service not later than 150 days prior to the opening date of the Plan Conference, and participants in the Plan shall submit their proposed changes through their Official State Agency.

(c) The name of the proponent shall be indicated on each proposed change when submitted. Each proposal should be accompanied by a brief supporting statement.

(d) The Service will notify all persons on the NPIP mailing lists concerning the dates and general procedure of the conference. Hatchery and dealer participants will be reminded of their privilege to submit proposed changes and to request copies of all the published proposed changes.

(e) The proposed changes, together with the names of the proponents and supporting statements, will be compiled by the Service and issued in processed form. When two or more similar changes are submitted, the Service will endeavor to unify them into one proposal acceptable to each proponent. Copies will be distributed to officials of the Official State Agencies cooperating in the NPIP. Additional copies will be made available for meeting individual requests.

§ 147.45 Official Delegates.

Each cooperating State shall be entitled to one official delegate for each of the programs prescribed in Subparts B, C, D, and E of Part 145 of this chapter in which it has one or more participants at the time of the Conference. The official delegates shall be elected by a representative group of participating industry members and be certified by the Official State Agency. It is recommended but not required that the official delegates be Plan participants. Each official delegate shall endeavor to obtain, prior to the Conference, the recommendations of industry members of his State with respect to each proposed change.

§ 147.46 Committee Consideration of Proposed Changes.

(a) The following four committees shall be established to give preliminary consideration to the proposed changes falling in their respective fields:
  (1) Egg-type chickens.
  (2) Meat-type chickens.
  (3) Turkeys.
  (4) Waterfowl, exhibition poultry, and game birds.

(b) Each official delegate shall be appointed a voting member in one of the committees specified in paragraph (a) of this section.

(c) Since several of the proposals may be interrelated, the committees shall consider them as they may relate to others, and feel free to discuss related proposals with other committees.

(d) The committees shall make recommendations to the conference as a whole concerning each proposal. The committee report shall show any proposed change in wording and the record of the vote on each proposal, and suggest an effective date for each proposal recommended for adoption. The individual committee reports shall be submitted to the chairman of the conference, who will combine them into one report showing, in numerical sequence, the committee recommendations on each proposal.

(e) The committee meetings shall be open to any interested person. Advocates for or against any proposal should feel free to appear before the appropriate committee and present their views.

§ 147.47 Conference Consideration of Proposed Changes.

(a) The chairperson of the conference shall be a representative of the Department.

(b) At the time designated for voting on proposed changes by the official delegates, the chairperson of the General Conference Committee and the four committee chairpersons shall sit at the speaker's table and assist the chairperson of the conference.

(c) Each committee chairperson shall present the proposals which his/her committee approves or recommends for adoption as follows: "Mr. Chairman. The committee for Egg-type chickens recommends the adoption of Proposal No. ———, for the following reasons (stating the reasons): I move the adoption of Proposal No. ———." A second will then be called for. If the recommendation is seconded, discussion and a formal vote will follow.

(d) Each committee chairman shall present the proposals which his committee does not approve as follows: "Mr. Chairperson. The Committee for Egg-type chickens does not approve Proposal No. ———." The chairperson will then ask if any official delegate wishes to move for the adoption of the proposal. If moved and seconded, the proposal is subject to discussion and voted. If there is no motion for approval, or if moved but not seconded, there can be no discussion or vote.

(e) Discussion on any motion must be withheld until the motion has been properly seconded, except that the delegate making the motion is privileged, if he/she desires, to give reasons for his/her motion at the time of making it. To gain the floor for a motion or for discussion on a motion, the official delegate in the case of a motion, or anyone in case of discussion on a motion, shall rise, address the chair, give his/her name and State, and be recognized by the chair before proceeding further. While it is proper to accept motions only from official delegates and to limit voting only to such delegates, it is, however, equally proper to accept discussion from anyone interested. To conserve time, discussion should be pointed and limited to the pertinent features of the motion.

(f) Proposals that have not been submitted in accordance with § 147.44 will be considered by the Conference only with the unanimous consent of the General Conference Committee. Any such proposals must be referred to the appropriate committee for consideration before being presented for action by the Conference.

(g) Voting will be by States, and each official delegate, as determined by § 147.45, will be allowed one vote on each proposal pertaining to the program prescribed by the subpart which he/she represents.

(h) A roll call of States for a recorded vote will be used when requested by a delegate or at the discretion of the chairperson.

(i) All motions on proposed changes shall be for adoption.

(j) Proposed changes shall be adopted by a majority vote of the official delegates present and voting.

(k) The Conference shall be open to any interested person.

§ 147.48 Approval of Conference Recommendations by the Department.

Proposals adopted by the official delegates will be recommended to the Department for incorporation into the provisions of the NPIP. The Department reserves the right to approve or disapprove the recommendations of the Conference as an integral part of its sponsorship of the National Poultry Improvement Plan.

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